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[Reprinted from SCIENCE, May 30, 1930, Vol. 71, No. 1848, p. 564]

DIRECT TRANSMISSION OF HUMAN TRACHOMA TO THE MONKEY

BY PETER K. OLITSKY AND JOSEPH R. TYLER

(From the Laboratories of The Rockefeller Institute for Medical Research)

In previous notes we drew attention to the successful transmission of experimental trachoma from monkey to monkey by (*a*) simple caging together of infected and uninfected *Macacus rhesus*,[1] (*b*) by repeated swabbing of the normal conjunctivae with the secretions from the experimental lesions in *Macacus rhesus*[2] and (*c*) by the repeated instillation of cultures of *Bact. granulosis* into the conjunctival sac of normal rhesus with subsequent massage of the eyelid.[2] The last method is, of course, not an example of monkey to monkey transmission except in respect to the principle of eye to eye conveyance.

In this note we are reporting two instances of direct transmission of trachoma by means of secretions from human cases to normal *Macacus rhesus*, which showed, previously to the swabbings, smooth conjunctivae. For the materials and effective cooperation we are indebted to Dr. Martin Cohen, of New York.

The cases consisted of two white persons residing in New York. Case A had suffered from trachoma for ten years. The lesions were characteristic, consisting of granulations, extensive scar formation and pannus. Case B had suffered from the active disease for two years. The lesions consisted also of granulations, scars and pannus.

The secretions from each case were taken on cotton swabs and transferred directly, by gentle rubbing, to the smooth conjunctivae of each of three monkeys. Nine swabbings were made from man to monkey in Case A and seven in Case B.

Thirteen days after the last swabbing from Case A, one monkey showed granular lesions of experimental trachoma, and thirteen days after the last transfer from Case B the three respective monkeys presented typical granulomatous changes.

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[1] J. R. Tyler, SCIENCE, 70: 612, 1929.

[2] P. K. Olitsky and J. R. Tyler, SCIENCE, 71: 263, 1930.

Conjunctival tissue was removed from Case A for curative purposes by Dr. Cohen and employed for direct subconjunctival injections in three further normal *Macacus rhesus* presenting smooth conjunctivae. Within four to ten days, all three developed granular lesions of experimental trachoma. The excised tissue was also employed for bacteriological study. Cultures of *Bact. granulosis* were isolated, and these when injected subconjunctivally into three normal monkeys induced in all experimental trachomatous changes in nine to eleven days.

Finally, granulomatous tissue removed from the monkey infected by swabbing from Case A yielded cultures of *Bact. granulosis.*

The direct transmission of trachoma to monkeys has already been effected by several investigators,[3] but the present experiments are the first in which both transmission and the isolation of *Bact. granulosis* have been successful with the same trachomatous material.

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[3] For literature see: H. Noguchi, *Jour. Exper. Med.*, Supplement No. 2, 1928, xlviii; and V. Morax and P. J. Petit, "Le Trachome," Paris, 1929.

[Reprinted from the PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE, May, 1930, Vol. 27, No. 8, pp. 813–814]

FURTHER OBSERVATIONS ON HEMAGGLUTINATION BY TUMOR EXTRACTS

BY K. LANDSTEINER

(From the Laboratories of The Rockefeller Institute for Medical Research)

The agglutinating substance found in tumor extracts[1] was not demonstrable in any appreciable amount in various organs of mice and rats, viz., liver, kidney, brain, spleen, heart. A distinctly positive reaction was obtained, especially in cysteine solutions, with muscle of rat, calf, and pig, and with the uteri of mice. Tumor and embryonic tissues were tested after drying. The dried material retains its activity for at least several weeks. The agglutinating substance of the tumor proved to be active after filtration through Berkefeld filters of a neutral, cysteine-containing solution. The active substance is absorbed by rabbit blood.

When extracts in 0.9% saline solution were heated at 55° for 20 minutes the activity was lost. This is evidently due to oxidation since in a saline solution containing 1% cysteine the agglutinating property persists and this is also the case when such solutions are brought to a boil or kept in boiling water, e.g., for 15 minutes. The substance was found to be still active when a water extract was heated to boiling, although the results were irregular. The activity of saline extracts which had been inactivated by boiling for a short time could be reactivated on the addition of a neutralized solution of cysteine to give a final concentration of 0.5%, and the same restitution effect was observed with extracts which had become inactive upon standing in the room for several hours.

The properties of the active substance suggested attempts to concentrate it. This could be done in the following manner: 6 gm. of rat sarcoma were ground with the addition of 5 cc. of water, and the suspension was poured into 25 cc. of boiling water, slightly acidulated with 3 drops of 5% acetic acid. After being heated to boiling, the suspension was cooled and coagulated protein removed by spinning. The supernatant fluid was neutralized and evaporated by vacuum *distillation. The apparatus used for distillation was filled with pure hydrogen which was passed through 2 washbottles with alkaline solution* of pyrogallol and a third bottle containing water. After evaporation to a small volume the water was completely removed in a well evacuated desiccator over P_2O_5. The dry material was then taken up in 5 cc. saline solution containing 0.2% cysteine. This solution had a stronger activity than the original tumor extract and agglutinated rabbit blood instantaneously. Moreover, in contrast to the original extract it agglutinated also sheep, horse, and guinea pig blood.

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[1] Landsteiner, K., PROC. SOC. EXP. BIOL. AND MED., 1928, xxvi, 134.

[Reprinted from The Journal of Experimental Medicine, July 1, 1930,
Vol. 52, No. 1, pp. 23–38]

STUDIES IN THE BLOOD CYTOLOGY OF THE RABBIT

II. Consecutive Erythrocyte and Hemoglobin Observations on Groups of Normal Rabbits

By LOUISE PEARCE, M.D., and ALBERT E. CASEY, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, April 14, 1930)

In the first paper of this series dealing with the blood cytology of the rabbit, the results of 1110 blood counts on 174 normal male rabbits were considered primarily from the standpoint of the numerical variations of the various classes of cells and of the hemoglobin content in individual determinations (1). Other phases of this investigation have included repeated observations on groups of normal and diseased rabbits for extended periods of time. In the case of normal animals, the study was undertaken chiefly for two reasons. Firstly, information was desired concerning the character of the spontaneous fluctuations in the numbers of blood cells under prolonged conditions of undisturbed indoor life with the idea that eventually some relationship might be found between these variations and certain environmental conditions, as was demonstrated with the weights of organs and the amount and degree of change of the total sunlight (2). Secondly, it was considered essential to have information of this nature as a background for whatever changes might be found in association with certain subacute or chronic experimental conditions, as for example, *Treponema pallidum* infection. Five groups of rabbits, 4 of 10 and 1 of 5 animals, were followed 35, 13, 8, 29, and 26 weeks respectively, covering a period of 20 months from October, 1927 to July, 1929. The group examinations numbered 111, in which 973 individual counts are represented.

The results of this study which have been analyzed statistically, will be reported in the present and subsequent papers. For the time being, the principal interest centers in the consecutive mean values of the various classes of cells considered from the standpoint of the trends of numerical levels shown by each animal group. In the present paper, the erythrocytes and the hemoglobin content are considered; other papers will deal with the total white cells and their division into granular and non-granular cells (3), with the neutrophiles (pseudo-eosinophiles), the basophiles, and the eosinophiles (4), and with the lymphocytes and the monocytes (5). The results will ultimately be analyzed from the standpoint of the relationships of the various classes of cells as shown by their correlation coefficients and in addition, they will be referred to in connection with the relation of the pre-inoculation blood picture to the reaction of the host to disease agents.

Materials and Methods

The rabbits employed were representative of those used in other experiments carried out in this laboratory; all were male animals approximately 6 to 8 months old. The ordinary brown and grey type predominated but the type described as the Flemish cross or mixture was also represented and there were a few black animals. Each rabbit was separately caged in a well lighted (sunlight), well ventilated room; the diet throughout the period of observation consisted of hay, oats, and cabbage.

The results reported are based upon 111 group examinations carried out at weekly intervals; in a few instances, the counts were made at shorter or longer intervals. The total number of individual blood counts was 973. The observations were made on 45 rabbits comprising 4 groups of 10 and 1 group of 5 animals:

Group	Number of rabbits	Number of examinations	Number of counts	First count	Last count
I	10	35	350	Oct. 24, 1927	June 20, 1928
II	10	13	130	Mar. 29, 1928	June 19, 1928
III	10	8	80	Sept. 20, 1928	Nov. 22, 1928
IV	10	29	283	Nov. 27, 1928	June 18, 1929
V	5	26	130	Dec. 29, 1928	June 21, 1929
	45	111	973		

A uniform routine with respect to the time and the method of collection and examination of the blood was followed as previously described (1). In the case of Group I, half of the animals were examined on one day and half on the following day of each week; all animals in each of the other groups were examined on the same day. The differential white blood determinations were made with the supravital neutral red technic; 100 cells were counted in each specimen.

In the case of Group I, additional specimens (10 cc.) of blood were taken for chemical examination. During the first 2 months of the experiment, this procedure was carried out at weekly intervals; from January to April, the period was extended to 2 weeks, and the last bleeding on May 17 was made 4 weeks after the preceding one. These bleedings always followed the blood counts by 1 or 2 days in order that as long a time as possible would elapse before the next blood count.

In the analysis of results, absolute numbers of cells per cubic millimeter of blood have been used. The curves obtained by plotting the actual group means were smoothed by the formula $\frac{a + 2b + c}{4}$, the initial value of each curve being represented by $\frac{2b + c}{3}$ and the final value by $\frac{a + 2b}{3}$. The coefficients of variation of the means have also been smoothed by this formula. The smoothed mean values have been compared, in the form of percentage deviations, with the mean numbers of cells found in 1110 normal blood counts (1). For convenience, certain figures used in this comparison as so-called standard values differ slightly from the actual results obtained, as shown in the following tabulation:

	Actual values	Values used
	per cmm.	*per cmm.*
Red blood cells	5,198,000	5,200,000
Hemoglobin	63%	63%
White blood cells	9562	9560
Neutrophiles	4341	4340
Basophiles	950	950
Eosinophiles	214	215
Lymphocytes	3045	3050
Monocytes	1000	1000
Total granular cells	5504	5505
Total non-granular cells	4045	4050

It should be pointed out that the results of the first 3 groups of animals here reported representing 560 counts, appear among the 1110 counts of the above tabulation; the 413 counts of the 4th and 5th groups, on the other hand, are not included in the large group.

The figures as given include all data. No count has been omitted because of the occurrence of such conditions as snuffles or ear canker in certain animals at some time during the period of observation, but these instances will be referred to in the discussion of the results.

RESULTS

The results pertaining to the consecutive weekly erythrocyte counts and the hemoglobin contents obtained in 5 groups of normal rabbits

TABLE I

Group I—10 Rabbits. Consecutive Values for Erythrocytes and Hemoglobin

Date	Red blood cells (000 omitted)			Hemoglobin		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
1927-28	*per cmm.*	*per cmm.*	*per cent*	*per cent*	*per cent*	*per cent*
Oct. 24*	5420 ± 192	901	16.62	54 ± 2.1	10	18.52
Nov. 1	5539 ± 42	199	3.59	49† ± 0.4	2	4.08
Nov. 8**	4991 ± 79	372	7.45	55 ± 1.4	7	12.73
Nov. 15	5039 ± 92	433	8.59	59 ± 1.3	6	10.17
Nov. 22	5204 ± 130	611	11.74	52 ± 1.3	6	11.54
Nov. 29	5351 ± 76	355	6.63	63 ± 1.3	6	9.52
Dec. 6	5323 ± 229	576	10.82	63 ± 2.1	10	15.87
Dec. 13	5075 ± 143	670	13.20	60 ± 1.4	7	11.67
Dec. 20	5032 ± 170	799	15.88	60 ± 1.4	7	11.67
Dec. 27	5296 ± 118	555	10.48	59 ± 1.4	7	11.86
Jan. 3	5286 ± 129	605	11.45	57 ± 2.3	11	19.30
Jan. 10	4889 ± 115	540	11.05	64 ± 1.4	7	10.94
Jan. 17	4859 ± 182	851	11.81	61 ± 1.4	7	11.48
Jan. 24	5513 ± 126	591	10.72	67 ± 1.4	7	10.45
Jan. 31	5399 ± 176	825	15.28	64 ± 1.7	8	12.50
Feb. 7	5474 ± 145	679	12.40	68 ± 1.3	6	8.82
Feb. 14	5204 ± 153	719	13.82	68 ± 1.3	6	8.82
Feb. 21	5336 ± 156	730	13.68	71 ± 1.3	6	8.45
Feb. 28	5312 ± 178	834	15.70	74 ± 1.0	5	6.76
Mar. 6	5158 ± 101	475	9.21	69 ± 1.7	7	10.14
Mar. 13	5451 ± 155	725	13.30	63 ± 1.3	6	9.52
Mar. 20	5024 ± 116	544	10.83	67 ± 1.3	6	8.96
Mar. 27	5229 ± 200	936	17.90	72 ± 2.1	10	13.89
Apr. 3	4838 ± 134	628	12.98	66 ± 1.9	9	13.64
Apr. 10	5273 ± 96	451	8.55	68 ± 2.1	10	14.71
Apr. 17	5455 ± 83	388	7.11	64 ± 1.0	5	7.81
Apr. 24	5050 ± 180	844	16.71	58 ± 1.9	9	15.52
May 1	5310 ± 148	696	13.11	64 ± 1.4	7	10.94
May 8	5560 ± 117	549	9.87	66 ± 2.3	11	16.67
May 15	5188 ± 110	515	9.93	66 ± 1.7	8	12.12
May 22	5335 ± 205	962	18.03	66 ± 2.1	10	15.15
May 29	5597 ± 162	761	13.60	64 ± 1.0	8	12.50
June 5	6080 ± 205	959	15.77	66 ± 1.3	6	9.09
June 12	5730 ± 110	518	9.04	68 ± 1.9	9	13.24
June 19	5779 ± 111	522	9.03	69 ± 0.8	4	5.80
Mean	5303 ± 30	263	4.96	64 ± 0.7	6	8.59
Minimum	4838			52		
Maximum	6080			74		

* October 24 and 26.

** November 4 and 9.

† Mean of 5 specimens.

TABLE II

Group II—10 Rabbits. Consecutive Values for Erythrocytes and Hemoglobin

Date	Red blood cells (000 omitted)			Hemoglobin		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
1928	*per cmm.*	*per cmm.*	*per cent*	*per cent*	*per cent*	*per cent*
Mar. 29	4912 ± 87	407	8.29	66 ± 1.3	6	9.09
Apr. 3	4899 ± 69	323	6.59	65 ± 1.4	4	6.15
Apr 10	4842 ± 62	289	8.84	66 ± 1.0	5	4.55
Apr. 17	5105 ± 163	762	14.93	64 ± 1.7	7	10.94
Apr. 24	5197 ± 83	387	7.45	60 ± 1.7	7	11.67
May 1	5169 ± 139	653	12.63	64 ± 1.7	7	10.94
May 8	5348 ± 119	558	10.43	63 ± 1.3	6	9.52
May 15	5047 ± 85	400	7.93	63 ± 1.7	6	9.52
May 22	5242 ± 96	450	8.58	66 ± 1.7	7	10.61
May 29	5270 ± 107	500	9.49	63 ± 1.7	7	11.11
June 5	5648 ± 116	523	9.26	69 ± 1.3	6	8.70
June 12	5329 ± 123	575	10.79	61 ± 1.3	6	9.84
June 19	5151 ± 90	421	8.17	64 ± 1.7	8	12.50
Mean	5166 ± 39	209	4.05	64 ± 0.4	2	3.13
Minimum	4899			60		
Maximum	5648			69		

TABLE III

Group III—10 Rabbits. Consecutive Values for Erythrocytes and Hemoglobin

Date	Red blood cells (000 omitted)			Hemoglobin		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
1928	*per cmm.*	*per cmm.*	*per cent*	*per cent*	*per cent*	*per cent*
Sept. 20	5039 ± 34	161	3.20	70 ± 1.7	8	11.43
Sept. 28	5129 ± 51	239	4.66	56 ± 1.0	5	8.93
Oct. 10	5075 ± 23	106	2.09	70 ± 1.0	5	7.14
Oct. 19	5116 ± 23	107	2.09	71 ± 1.7	8	11.27
Nov. 2	5108 ± 51	240	4.70	56 ± 1.0	5	8.93
Nov. 9	5108 ± 32	151	2.96	66 ± 1.4	7	10.61
Nov. 16	5091 ± 41	191	3.75	65 ± 1.0	7	10.77
Nov. 22	5252 ± 33	153	2.91	68 ± 1.3	6	8.82
Mean	5115 ± 15	62	1.21	65 ± 1.4	6	9.23
Minimum	5039			56		
Maximum	5252			71		

TABLE IV

Group IV—10 Rabbits. Consecutive Values for Erythrocytes and Hemoglobin

Date	Red blood cells (000 omitted)			Hemoglobin		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
1928-29	*per cmm.*	*per cmm.*	*per cent*	*per cent*	*per cent*	*per cent*
Nov. 27	5694 ± 141	660	11.59	68 ± 2.1	10	14.83
Dec. 4	5274 ± 107	502	9.52	64 ± 1.4	7	10.28
Dec. 11	5549 ± 127	595	10.72	67 ± 1.9	9	13.33
Dec. 18	5375 ± 53	247	4.60	61 ± 1.2	6	9.50
Dec. 26	5285 ± 83	389	7.35	69 ± 1.7	8	11.31
Jan. 2	5274 ± 107	504	9.55	64 ± 2.0	9	14.68
Jan. 8	4839 ± 65	306	6.32	55 ± 1.2	6	10.18
Jan. 15	4860 ± 62	291	5.98	59 ± 1.3	6	10.61
Jan. 22	4810 ± 44	206	4.27	59 ± 1.9	9	15.11
Jan. 29	4941 ± 68	317	6.42	54 ± 1.0	5	8.25
Feb. 5	4705 ± 69	322	6.84	58 ± 1.2	6	9.81
Feb. 13	5028 ± 57	266	5.29	60 ± 1.1	5	8.60
Feb. 19	4914 ± 86	405	8.24	61 ± 1.3	6	10.18
Feb. 26	5034 ± 53	247	4.90	60 ± 1.2	6	9.16
Mar. 12	4897 ± 52	244	4.98	63 ± 1.6	8	11.85
Mar. 19	4943 ± 66	311	6.28	60 ± 0.9	4	7.05
Mar. 26	4875 ± 61	284	5.83	59 ± 0.8	4	6.75
Apr. 2	4820 ± 35	165	3.42	60 ± 1.2	5	9.00
Apr. 9	4774 ± 55	256	5.35	58 ± 1.1	5	8.68
Apr. 16	4849 ± 62	292	6.02	62 ± 1.4	7	10.50
Apr. 23	4874 ± 49	229	4.69	61 ± 1.1	5	8.75
Apr. 30	4806 ± 33	155	3.22	64 ± 1.1	5	7.94
May 7	4907 ± 40	189	3.86	64 ± 0.7	3	5.06
May 14	4816 ± 28	123	2.55	62 ± 1.6	7	11.34
May 21	4808 ± 40	177	3.68	64 ± 1.4	6	9.91
May 28	4843 ± 65	287	5.92	59 ± 1.7	8	13.05
June 4	4843 ± 56	249	5.14	66 ± 1.8	8	11.82
June 11	4936 ± 38	171	3.46	63 ± 2.4	11	17.00
June 18	4888 ± 47	197	4.03	56 ± 1.4	6	10.16
Mean	4981 ± 31	245	4.93	61 ± 0.5	4	5.75
Minimum	4705			54		
Maximum	5694			69		

observed 8 to 35 weeks are presented in Tables I to V in the form of group means, together with the probable errors of the means, the standard deviations, and the coefficients of variation. The series of

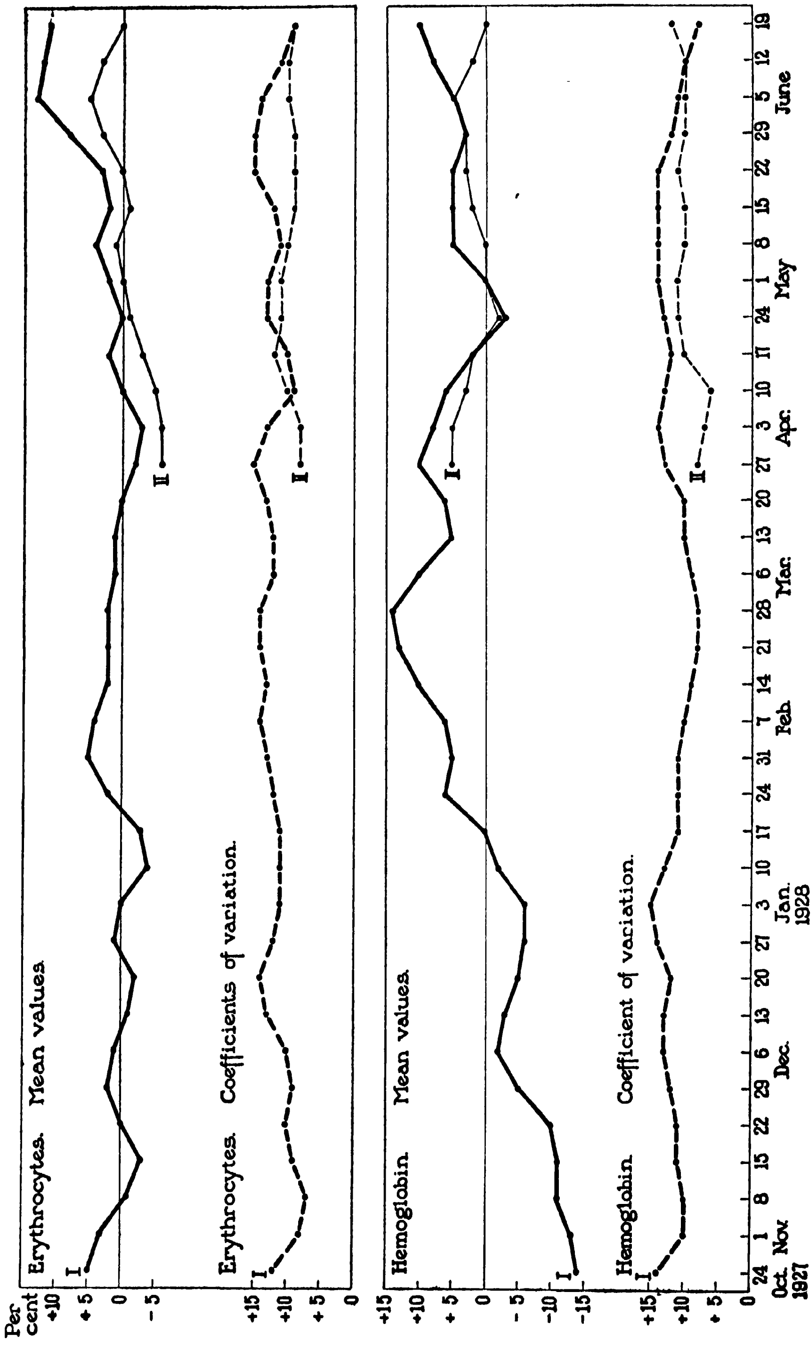
TABLE V

Group V—5 Rabbits. Consecutive Values for Erythrocytes and Hemoglobin

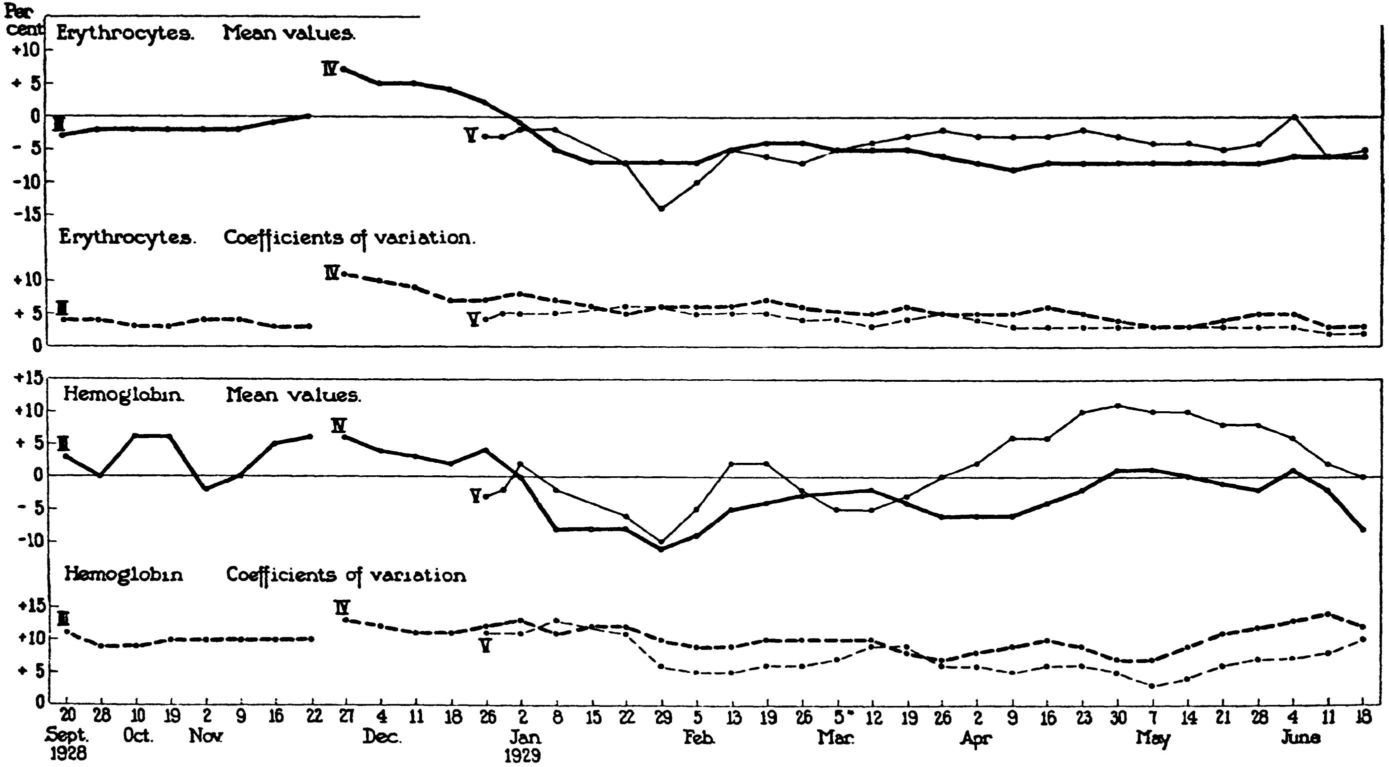
Date	Red blood cells (000 omitted)			Hemoglobin		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
1928–29	*per cmm.*	*per cmm.*	*per cent*	*per cent*	*per cent*	*per cent*
Dec. 29	5100 ± 57	190	2.73	60 ± 1.8	6	10.00
Dec. 31	4920 ± 90	297	6.04	62 ± 2.4	8	12.90
Jan. 3	5224 ± 84	279	5.34	64 ± 1.5	5	7.81
Jan. 10	5108 ± 65	214	4.19	64 ± 3.3	11	17.19
Jan. 24	4904 ± 82	271	5.53	57 ± 1.8	6	10.53
Jan. 31	4384 ± 97	323	7.37	56 ± 0.6	2	3.57
Feb 7	4592 ± 75	249	5.42	58 ± 0.9	3	5.17
Feb. 15	5216 ± 54	178	3.41	67 ± 0.9	3	4.48
Feb. 21	4730 ± 106	352	7.44	64 ± 1.2	4	6.25
Mar. 1	4850 ± 35	117	2.41	61 ± 0.6	3	4.92
Mar. 8	4894 ± 78	258	5.27	61 ± 1.2	4	6.56
Mar. 15	5072 ± 37	124	2.44	58 ± 1.8	6	10.34
Mar. 22	4990 ± 47	156	3.13	61 ± 1.8	6	9.84
Mar. 29	5228 ± 86	286	5.47	64 ± 0.9	3	4 69
Apr. 5	4940 ± 58	192	3.89	62 ± 1.2	4	6.45
Apr. 12	5078 ± 50	164	3.23	69 ± 0.9	3	4.35
Apr. 19	5030 ± 33	108	2.15	66 ± 1.2	4	6.06
Apr. 26	5078 ± 57	189	3.72	70 ± 1.5	5	7.14
May 3	5128 ± 32	105	2.05	71 ± 0.9	3	4.23
May 10	4882 ± 60	198	4.06	68 ± 0.6	2	2.94
May 17	4984 ± 39	130	2.61	70 ± 0.6	2	2.86
May 24	4966 ± 49	161	3.24	67 ± 1.5	5	7.46
May 31	4952 ± 42	138	2.79	67 ± 1.5	5	7.46
June 7	5050 ± 42	140	2.77	70 ± 1.5	5	7.14
June 14	4756 ± 29	96	2.02	60 ± 1.2	4	6.67
June 21	5010 ± 33	108	2.16	64 ± 2.1	7	10.94
Mean	4964 ± 25	188	3.79	64 ± 0.5	4	6.25
Minimum	4384			56		
Maximum	5228			71		

curves in Text-figs. 1 and 2 are drawn as percentage deviations of the smoothed means from the standard values given above; other curves represent the smoothed values of the coefficients of variation of the means.

By using mean values to describe the results obtained, the trends or



TEXT-FIG. 1. Mean values for consecutive red blood cell and hemoglobin determinations as percentage deviations from standard values. 1927–28.



TEXT-FIG. 2. Mean values for consecutive red blood cell and hemoglobin determinations as percentage deviations from standard values. 1928–29.

changes characteristic of the group as a whole are shown. It is this feature of the results rather than the variations of individual animals which at present we wish to emphasize.

DISCUSSION AND SUMMARY

In discussing the results of repeated observations on the erythrocytes and hemoglobin content of the peripheral blood of normal rabbits over long periods of time, it will be convenient to consider in chronological order the findings obtained in each of the five groups.

Group I, comprising 10 rabbits, was examined weekly from October 24, 1927 to June 21, 1928 (Table I). The mean numbers of red cells in 35 examinations were found to be comparatively constant.

The fluctuations in the curve of the percentage deviations of the smoothed means from a standard value of 5,200,000 cells per cubic millimeter (Text-fig. 1) do not vary more than 5 per cent above or below this figure until May and June when the curve rises to the 10 to 13 per cent level. In general, the curve describes 3 major swings, each occupying a period of approximately 3 months. From the October level of 5 per cent above the base line, there is an irregular fall to 4 per cent below it on January 10. During the succeeding 3 weeks, the curve rises abruptly to its initial level and very gradually descends to slightly below the base line (March 27 and April 3). During the last 3 months the curve rises to the highest levels observed although at its termination, its trend is again downward. The coefficients of variation for the red cells means (Table I) are fairly uniform, the curve of the smoothed values varying about the 10 per cent level (Text-fig. 1). The most irregular portion of the curve is its last quarter and this phase accompanied an augmentation of mean values. It will be noted that the changes in the trend of the red cell means at the end of January and the first of April are, in both instances, preceded by similar changes in the coefficient curve, at a 2 or 3 weeks' interval.

The range of mean hemoglobin values of Group I (Table I) was considerably greater than that of the erythrocyte means. The curve of these values in terms of the percentage variations of the smoothed means from a standard of 63 per cent fluctuates within a total range of 28 per cent, that is, 14 per cent above and below the base line (Text-fig. 1).

This curve can be divided into 3 major swings which, however, are not parallel with those of the red cells. From an initial low level the curve rises to 14 per cent above the base line by the end of February; the trend of the red cell curve in the opposite direction ended the middle of January and for the next 3 observations, the direction of both curves is upward. From the end of February to the last of April, the hemoglobin curve is generally falling, while in May and June its general movement is again in an upward direction. In March, both the hemoglobin and red cell curves are falling and from the middle of April to the end of June both are rising. The coefficients of variation of the hemoglobin means (Table I) are comparable as far as order of magnitude is concerned, to those of the red cell means. From the curve representing the smoothed coefficients (Text-fig. 1) it will be noted that small values prevailed during January and February in which months the means were considerably increased. In like manner, the second period of higher hemoglobin mean values in May and June was characterized by a steady decline of the coefficient curve.

The second group of 10 rabbits (Group II) was examined during the last 3 months of the observation period of Group I (Table II). The values for the mean numbers of erythrocytes and for the mean hemoglobin contents occupy slightly lower levels than those for Group I (Text-fig. 1), but it will be noted that the curves representing these values are remarkably like those of Group I in general outline, the only important exception being in the case of the hemoglobin means during the last 2 weeks' observations.

It should be noted that in the corresponding portion of the curve representing the coefficients of variation of these means, an abrupt upward turn occurs indicating that the sharp fall in the mean values is due to certain individual findings and is not characteristic of the group as a whole. The coefficients of variations of both erythrocyte and hemoglobin means of Group II (Table II) are generally somewhat smaller than those of Group I as is illustrated by the curves shown in Text-fig. 1.

The third group of 10 rabbits (Group III) was examined in September, October, and November of 1928. The erythrocytes and the hemoglobin means, together with their coefficients of variations are given in Table III and the curves representing these values appear in Text-fig. 2.

In the case of the red blood cells, the means were extremely regular at a level slightly below the standard value while the curve of their smoothed coefficients of variation is steadily maintained at the 3 to 4 per cent level. The hemoglobin means, on the other hand, were less uniform and their curve describes several fluctuations which, however, are of a much smaller magnitude than those of Group I. In contrast to these variations, however, the curve of the smoothed coefficients of variation of these means is almost a straight line at the 10 per cent level, a value slightly smaller than that which generally obtained in Group I.

Examination of the fourth group of 10 rabbits was begun in November, 1928 and of the fifth group comprising 5 rabbits in December, 1928; both groups were observed to the end of June, 1929. The erythrocyte and hemoglobin means and their coefficients of variations are given in Tables IV and V, and the curves illustrating these values in Text-fig. 2.

In the case of Group IV, the red cell curve which shows practically no irregularities, describes during the first 2 months' observation a steady downward trend from an initial 7 per cent above to 7 per cent below the standard value; during the remainder of the experiment this low level is almost perfectly maintained. The curve of the smoothed coefficients of variation of the red cell means has a similar form; from an initial 11 per cent level, it falls to 5 per cent at the end of the second month and to 3 per cent in June.

The hemoglobin means of Group IV were much less uniform than the red cell values as was the case with the previous groups; the fluctuations of the curve representing these values (Text-fig. 2) vary between 6 per cent above to 11 per cent below the base line. During the first 2 months, the direction of the curve is downward, parallel to that of the red cells, but from this time onward, while the red cell means continue at a fairly constant level, the general trend of the hemoglobin means is upward. The rise is interrupted in March and again in June; on the first occasion there is also a fall in the number of red cells but on the second, the red cell level is unchanged. The smoothed coefficients of variation of the hemoglobin means for Group IV vary from 7 to 14 per cent (Text-fig. 2). Their curve is fairly stationary at the 11 to 12 per cent level during the first 2 months' observations; in subsequent months somewhat lower and more irregular values obtain, and in May and June a rise to 12 and 14 per cent is observed.

The curve representing the erythrocyte means of Group V differs but little from that of Group IV as shown in Text-fig. 2. It begins in December at approximately the same level which Group IV occupies at this time and ends at the same level in June. There is an initial abrupt fall to 14 per cent below the base line (end of January) which is followed by a sharp rise, and from then onward, a fairly constant level is maintained at 5 per cent below the standard value. During January and February, the general level of the curve is slightly lower than that of Group IV while in March, April, May, and June, the relation is reversed. On the whole, the curve is uniform and contains but two irregular portions, one the end of January when the downward trend is prolonged to the lowest level observed in any of the 5 groups, that is, 13 per cent below the standard value, and the other the first of June at which time there is a sharp but temporary upward movement. The curve representing the smoothed coefficients of variation of the red cell means which is extremely uniform (Text-fig. 2) occupies a general level slightly below that of Group IV. It describes a gradual fall from 6 to 2 per cent.

In the case of the hemoglobin content, the mean values of Group V were somewhat higher than those of Group IV, but the direction of the variations observed was essentially the same (Text-fig. 2). The curves representing these values are similar in general contour with parallel fluctuations but those of Group IV are somewhat more pronounced, their extremes being 10 per cent below and 11 per cent above the standard value. The level of the curve representing the smoothed coefficients of variation of the hemoglobin means of Group V is generally lower than that of Group IV, as was the case with the red cells (Text-fig. 2); the fluctuations in magnitude vary from 3 to 13 per cent. The general trend of this curve is similar to that of Group IV.

The great majority of the rabbits in these experiments were in excellent physical condition and gained in weight during the period of observation. There were two deaths, both in Group IV, which occurred toward the end of the experiment. Both animals had had marked snuffles and at autopsy, an extensive purulent exudate was found in the nasal passages and sinuses and in one rabbit, there was also a chronic diffuse interstitial nephritis. Snuffles and ear canker of various grades which were observed in certain rabbits will be referred to in a subsequent paper in connection with the discussion of the observations on the neutrophile cells (4).

There are certain features of these consecutive observations on the peripheral blood of normal rabbits which may now be briefly considered. In the first place, the weekly range of variation of the erythrocyte mean counts was considerably smaller than that of the hemoglobin means, a result which indicates that under the conditions of these experiments, the hemoglobin was the more labile of these two blood constituents. The technic employed may have contributed to this finding in that the error involved in a colorimetric method may be greater than in one of cell enumeration. With each specimen of blood, at least 3 hemoglobin readings were made and the mean value used; and in addition, duplicate readings with another instrument have always checked with those of our own. Secondly, it was found that a brief or a more sustained alteration in the erythrocyte means was not necessarily accompanied by a shift of the hemoglobin level in the opposite direction. In certain instances, such a change was observed, but in others, both values moved in the same direction and in still others, the hemoglobin means pursued a more or less protracted rise while the numbers of red cells were maintained at a fairly stationary level. Thirdly, the most irregular results with respect to both red cell and hemoglobin means were found in Group I and in addition, their standard deviations were on the whole, of a higher order of magnitude than those of the other groups (Tables I to V). The examinations on Group I were not all made on the same day, half the animals being examined on one day and half on the following day; in the case of the other groups, all animals were examined on the same day. As has been mentioned in the section on Materials and Methods, the rabbits of Group I were also subjected to additional bleedings for purposes of chemical examination. Although this procedure was carried out most frequently in the first 2 months of the experiment, there is no striking difference in the order of magnitude of the standard deviations of the means nor in the coefficients of variations during this period as compared with later months (Table I). It is probable, therefore, that the greater irregularities of mean values and the higher standard deviations noted in Group I were related to the divided time of the blood counts themselves or to some other factor rather than to the additional bleedings for chemical examination.

In the present consideration, the major changes in mean values as observed over several weeks or months have been emphasized rather than fluctuations occurring in comparatively brief periods. Analysis of the results from the standpoint of the ratio of the difference of the mean values to their probable errors indicates that the major changes observed in both the red cell and hemoglobin levels are of statistical significance, as shown by the following examples:

Group number	Red blood cells			Hemoglobin		
	Dates of mean values		Ratio	Dates of mean values		Ratio
I	Jan. 10	Jan. 24	3.65	Oct. 24	Jan. 10	2.94
	Apr. 3	June 19	5.44	Apr. 24	June 19	5.24
II	Mar. 29	May 8	2.96	Mar. 29	Apr. 24	2.73
	Apr. 3	June 12	3.05	Apr. 24	June 5	4.09
III	Sept. 20	Nov. 22	4.53	Sept. 20	Nov. 2	7.00
IV	Nov. 27	June 18	5.38	Nov. 27	June 18	4.80
	Feb. 26	Apr. 30	3.68	Dec. 11	Jan. 22	2.96
V	Dec. 29	Feb. 7	5.40	Jan. 24	Apr. 19	4.09
	Feb. 21	Apr. 26	3.25	Apr. 26	June 14	5.26

A striking feature of these results is the degree of parallelism shown by two groups of animals examined over the same period with respect to the fluctuations of erythrocytes and hemoglobin mean values. The curves in Text-figs. 1 and 2 show that the trend of values for one group of animals is almost always reflected in a similar trend for the other group.

Finally, these observations make it clearly evident that the results obtained on the groups of rabbits examined in one year may not be entirely similar to those obtained on other groups followed for similar periods in another year. In the case of both the erythrocytes and the hemoglobin, the mean values during 1927–28 were generally higher than those of 1928–29. Furthermore, there was a definite trend on the part of both red cell and hemoglobin means toward higher values in the spring and early summer months of the first year which were not observed in the following year. In the fall and early winter months of both years, on the other hand, the red cells tended toward smaller values; this was also the case for the hemoglobin in the second but not in the first year.

CONCLUSIONS

Observations are reported on the consecutive weekly erythrocyte counts and the hemoglobin contents of the peripheral blood in 5 groups of normal rabbits, comprising 45 animals, during a period of 20 months from October, 1927 to July, 1929. The duration of individual group examinations varied from 8 to 35 weeks. The results are analyzed on the basis of the weekly mean values of each group.

On the whole, the erythrocyte values were quite uniform within a narrow range of variation, while the hemoglobin content was comparatively irregular within a wider range of variation. The major changes in the levels of mean values of both the red cells and the hemoglobin, however, were found to be statistically significant.

The directions or trends in the levels of the erythrocyte and hemoglobin mean values did not necessarily move in opposite directions.

The general levels of the erythrocyte and hemoglobin mean values were not identical for two consecutive years, those of 1927–28 being higher than those of 1928–29.

The fluctuations of both red cell and hemoglobin mean values observed in one group of animals were also usually observed in another group examined during the same months.

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STUDIES IN THE BLOOD CYTOLOGY OF THE RABBIT

III. CONSECUTIVE TOTAL WHITE BLOOD CELL OBSERVATIONS ON GROUPS OF NORMAL RABBITS

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In the preceding paper of this series, experiments dealing with consecutive observations on the peripheral cytology of normal rabbits for prolonged periods of time were described and the results pertaining to the erythrocytes and the hemoglobin were reported (1). The observations were made on 5 groups of male rabbits, 4 of 10 and 1 of 5 animals, during a period extending from October, 1927 to July, 1929. In the present paper, the results relating to the total white cell counts are reported together with a consideration of these values from the standpoint of the total granular leucocytes—the neutrophiles (pseudoeosinophiles), the basophiles, and the eosinophiles—on the one hand, and the total non-granular cells—the lymphocytes and the monocytes—on the other.

Materials and Methods

Since the conduct of the experiments and the methods employed in analyzing the results obtained have previously been described (1), it will suffice here merely to state that in the great majority of cases the examination of the blood was carried out at weekly intervals. The differential counts which were made by means of the supravital neutral red technic, were based upon counts of 100 cells for each specimen.

The number of examinations on each group of rabbits was: Group I, 35; Group II, 13; Group III, 8; Group IV, 29; Group V, 26. The first 4 groups comprised 10, and the last, 5 animals.

RESULTS

The results on consecutive total white blood cell counts in 5 groups of normal rabbits observed 8 to 35 weeks are given in Tables I to V and

TABLE I

Group I—10 Rabbits. Consecutive Values for Total White Blood Cells and for Total Granular and Non-Granular Cells

Date	Total white blood cells			Granular: Neutrophiles, basophiles, and eosinophiles			Non-granular: Lymphocytes and monocytes		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
1927–28	*per cmm.*	*per cmm.*	*per cent*	*per cmm.*	*per cmm*	*per cent*	*per cmm.*	*per cmm.*	*per cent*
Oct. 24*	8825 ± 401	1879	21.29	5586 ± 261	1224	21.91	3240 ± 243	1138	35.12
Nov. 1	8245 ± 430	2015	24.44	5293 ± 304	1423	26.89	2953 ± 188	879	29.77
Nov. 8**	8030 ± 507	2376	29.59	4948 ± 283	1328	26.84	3082 ± 261	1222	39.65
Nov 15	8555 ± 275	1288	15.06	4910 ± 192	901	18.35	3645 ± 132	618	16.95
Nov. 22	9935 ± 601	2816	28.34	6522 ± 355	1666	25.54	3414 ± 224	1048	30.70
Nov. 29	9685 ± 747	3504	36.18	5494 ± 578	2710	49.33	4192 ± 265	1241	29.60
Dec. 6	8840 ± 463	2169	24.54	4987 ± 395	1852	37.14	3854 ± 177	831	21.56
Dec. 13	9130 ± 756	3546	38.84	4783 ± 393	1841	38.49	4348 ± 469	2198	50.55
Dec. 20	9690 ± 582	2729	28.16	6251 ± 417	1955	31.27	3439 ± 196	920	26.75
Dec. 27	9400 ± 380	1780	18.94	5983 ± 258	1209	20.21	3418 ± 200	936	27.38
Jan. 3	9800 ± 469	2200	22.45	5811 ± 254	1192	20.51	3990 ± 347	1628	40.80
Jan. 10	10240 ± 440	2065	20.17	5827 ± 338	1586	27.22	4392 ± 255	1195	27.21
Jan. 17	10375 ± 613	2873	27.69	5899 ± 496	2327	39.45	4454 ± 345	1616	36.28
Jan. 24	10380 ± 481	2253	21.70	5891 ± 277	1301	22.08	4478 ± 260	1217	27.18
Jan. 31	11081 ± 718	3367	30.39	6590 ± 520	2439	37.01	4466 ± 335	1571	35.18
Feb. 7	11095 ± 651	3053	27.52	6477 ± 544	2550	39.37	4601 ± 240	1123	24.41
Feb. 14	10920 ± 671	3146	28.81	6643 ± 554	2595	39.06	4270 ± 77	362	8.48
Feb. 21	12450 ± 504	2365	19.00	6545 ± 353	1654	25.27	5888 ± 246	1152	19.57
Feb. 28	11700 ± 913	4281	36.59	8052 ± 842	3950	49.06	3651 ± 65	303	8.30
Mar. 6	10455 ± 494	2316	22.15	6390 ± 329	1543	24.15	4051 ± 230	1078	26.61
Mar. 13	9910 ± 657	3079	31.07	6186 ± 505	2368	38.28	3715 ± 276	1296	34.89
Mar. 20	11270 ± 792	3711	32.93	7164 ± 760	3561	46.77	3647 ± 170	795	21.80

Mar. 27	10230 ± 499	2338	22.85	6062 ± 372	1742	28.74	4156 ± 235	1100	26.47
Apr. 3	10750 ± 620	2908	27.16	6202 ± 429	2013	32.46	4503 ± 397	1859	41.28
Apr. 10	12275 ± 679	3183	25.93	7271 ± 464	2174	29.90	4992 ± 221	1038	20.79
Apr. 17	11225 ± 543	2547	22.69	6385 ± 379	1779	27.86	4840 ± 255	1195	24.69
Apr. 24	10535 ± 718	3367	31.96	6522 ± 561	2629	40.31	4006 ± 315	1475	36.84
May 1	11240 ± 570	2670	23.75	6130 ± 303	1422	23.20	5098 ± 301	1411	27.68
May 8	11100 ± 726	3404	30.67	6644 ± 481	2255	33.94	4439 ± 309	1448	32.62
May 15	11605 ± 504	2364	20.37	6761 ± 388	1819	26.90	4845 ± 199	933	19.26
May 22	12085 ± 698	3273	27.08	7217 ± 570	2670	37.00	4869 ± 232	1088	22.35
May 29	12035 ± 901	4226	35.11	6443 ± 369	1732	26.88	5447 ± 594	2787	51.17
June 5	13590 ± 532	2495	18.36	7445 ± 312	1461	19.62	6144 ± 321	1504	24.48
June 12	12380 ± 797	3735	30.17	6656 ± 330	1547	23.24	5725 ± 474	2221	38.79
June 19	11455 ± 831	3895	34.00	6973 ± 497	2332	33.44	4482 ± 402	1883	40.90
Mean	10585 ± 146	1281	12.10	6269 ± 87	759	12.11	4307 ± 87	763	17.72
Minimum	8030			4783			2953		
Maximum	13590			8052			6144		

* October 24 and 26.

** November 4 and 9.

TABLE II

Group II—10 Rabbits. Consecutive Values for Total White Blood Cells and for Total Granular and Non-Granular Cells

Date	Total white blood cells			Granular: Neutrophiles, basophiles, and eosinophiles			Non-granular: Lymphocytes and monocytes		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
1928	*per cmm.*	*per cmm.*	*per cent*	*per cmm.*	*per cmm.*	*per cent*	*per cmm.*	*per cmm.*	*per cent*
Mar. 29	7095 ± 275	1290	18.18	4300 ± 257	1206	28.05	2783 ± 185	866	31.12
Apr. 3	8735 ± 422	1979	22.66	4745 ± 205	962	20.07	3990 ± 287	1345	33.71
Apr. 10	8445 ± 534	2504	29.65	4516 ± 346	1622	35.92	3917 ± 320	1498	38.24
Apr. 17	10150 ± 531	2487	24.50	5640 ± 352	1652	29.29	2405 ± 348	1631	36.20
Apr 24	9350 ± 525	2462	26.33	5430 ± 367	1720	31.68	3900 ± 240	1126	28.72
May 1	8040 ± 315	1477	18.37	4103 ± 209	978	23.84	3927 ± 237	1109	28.17
May 8	9085 ± 394	1845	20.31	5093 ± 299	1401	27.51	3992 ± 233	1092	27.35
May 15	7610 ± 579	2716	35.69	4285 ± 390	1829	42.68	3325 ± 276	1293	38.89
May 22	11120 ± 449	2107	18.95	6132 ± 351	1645	26.83	4988 ± 279	1308	26.22
May 29	9460 ± 702	3289	34.77	4847 ± 313	1465	30.22	4601 ± 416	1949	42.36
June 5	10230 ± 445	2088	20.41	5570 ± 317	1485	26.66	4660 ± 283	1325	28.43
June 12	9030 ± 557	2611	28.91	4772 ± 254	1190	25.20	4229 ± 323	1514	35.22
June 19	10355 ± 375	1756	16.96	5663 ± 267	1250	22.07	4692 ± 234	1097	23.38
Mean	9131 ± 208	1114	12.20	5007 ± 114	609	12.16	3958 ± 135	722	18.24
Minimum	7095			4103			2405		
Maximum	11120			6132			4988		

TABLE III

Group III—10 Rabbits. Consecutive Values for Total White Blood Cells and for Total Granular and Non-Granular Cells

Date	Total white blood cells			Granular: Neutrophiles, basophiles, and eosinophiles			Non-granular: Lymphocytes and monocytes		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
1928	*per cmm.*	*per cmm.*	*per cent*	*per cmm.*	*per cmm.*	*per cent*	*per cmm.*	*per cmm.*	*per cent*
Sept. 20	8040 ± 672	3152	39.20	5337 ± 355	1662	31.02	2683 ± 281	1319	49.16
Sept. 28	7030 ± 474	2223	31.62	3796 ± 176	826	21.76	3234 ± 292	1368	42.30
Oct. 10	7715 ± 487	2282	29.58	4779 ± 427	2000	41.68	2916 ± 99	464	15.91
Oct. 19	6985 ± 381	1785	25.55	3675 ± 118	553	15.05	3310 ± 207	969	29.27
Nov. 2	7560 ± 337	1581	20.91	4754 ± 204	955	20.09	2806 ± 101	473	16.86
Nov. 9	7885 ± 297	1393	17.67	4713 ± 292	1370	29.07	3173 ± 113	783	24.68
Nov. 16	6595 ± 270	1264	19.17	3940 ± 144	674	17.11	2655 ± 176	824	31.04
Nov. 22	7165 ± 314	1473	20.56	3922 ± 168	788	20.09	3243 ± 196	921	28.40
Mean	7372 ± 112	471	6.39	4365 ± 135	566	12.97	3003 ± 60	251	8.36
Minimum	6985			3675			2655		
Maximum	8040			5337			3310		

TABLE IV

Group IV—10 Rabbits. Consecutive Values for Total White Blood Cells and for Total Granular and Non-Granular Cells

Date	Total white blood cells			Granular: Neutrophiles, basophiles, and eosinophiles			Non-granular: Lymphocytes and monocytes		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
1928-29	*per cmm.*	*per cmm.*	*per cent*	*per cmm.*	*per cmm.*	*per cent*	*per cmm.*	*per cmm.*	*per cent*
Nov. 27	7755 ± 201	1605	20.69	4593 ± 265	1243	27.07	3162 ± 195	918	29.03
Dec. 4	7665 ± 357	1673	21.82	4453 ± 308	1445	32.44	3212 ± 162	762	23.72
Dec. 11	7245 ± 438	2054	28.34	4467 ± 308	1446	32.37	2771 ± 216	1012	36.51
Dec. 18	6660 ± 430	2014	30.23	3670 ± 198	926	25.23	2958 ± 291	1365	46.13
Dec. 26	6720 ± 357	1673	24.88	4192 ± 281	1319	31.46	2522 ± 143	679	26.91
Jan. 2	6340 ± 237	1111	17.52	4320 ± 209	979	22.66	2019 ± 81	390	19.32
Jan. 8	6125 ± 403	1890	30.86	3595 ± 326	1527	42.48	2530 ± 166	790	31.21
Jan. 15	6620 ± 270	1265	19.10	4087 ± 221	1035	25.31	2518 ± 96	458	18.20
Jan. 22	6645 ± 431	2021	30.41	4102 ± 274	1283	31.28	2543 ± 201	953	37.47
Jan. 29	8570 ± 462	2166	25.27	5510 ± 322	1509	27.37	3061 ± 179	851	27.80
Feb. 5	6535 ± 412	1932	29.56	4116 ± 325	1526	37.07	2419 ± 186	884	36.53
Feb. 13	8840 ± 550	2579	29.17	5978 ± 391	1833	30.66	2862 ± 207	980	34.24
Feb. 19	9890 ± 352	1648	16.66	7050 ± 319	1495	21.19	2800 ± 159	757	27.04
Feb. 26	9515 ± 418	1960	20.59	6364 ± 359	1683	26.44	3104 ± 141	673	21.67
Mar. 12	7785 ± 261	1224	15.71	4731 ± 186	873	18.44	3013 ± 195	922	30.61
Mar. 19	9305 ± 484	2267	24.36	6259 ± 423	1982	31.66	2046 ± 175	829	27.20
Mar. 26	8875 ± 501	2349	26.46	5926 ± 371	1741	29.37	2925 ± 237	1119	38.26
Apr. 2	8475 ± 520	2436	28.74	6058 ± 398	1865	30.78	2417 ± 218	1032	42.69
Apr. 9	9365 ± 343	1610	17.19	5686 ± 146	685	12.04	3596 ± 240	1136	46.98
Apr. 16	9360 ± 470	2204	23.54	5886 ± 326	1527	25.94	3474 ± 252	1291	37.16
Apr. 23	10185 ± 440	2062	20.24	6246 ± 254	1193	19.10	3887 ± 249	1276	32.82
Apr 30	8840 ± 347	1625	18.38	5912 ± 244	1144	19.36	2920 ± 214	1113	38.09

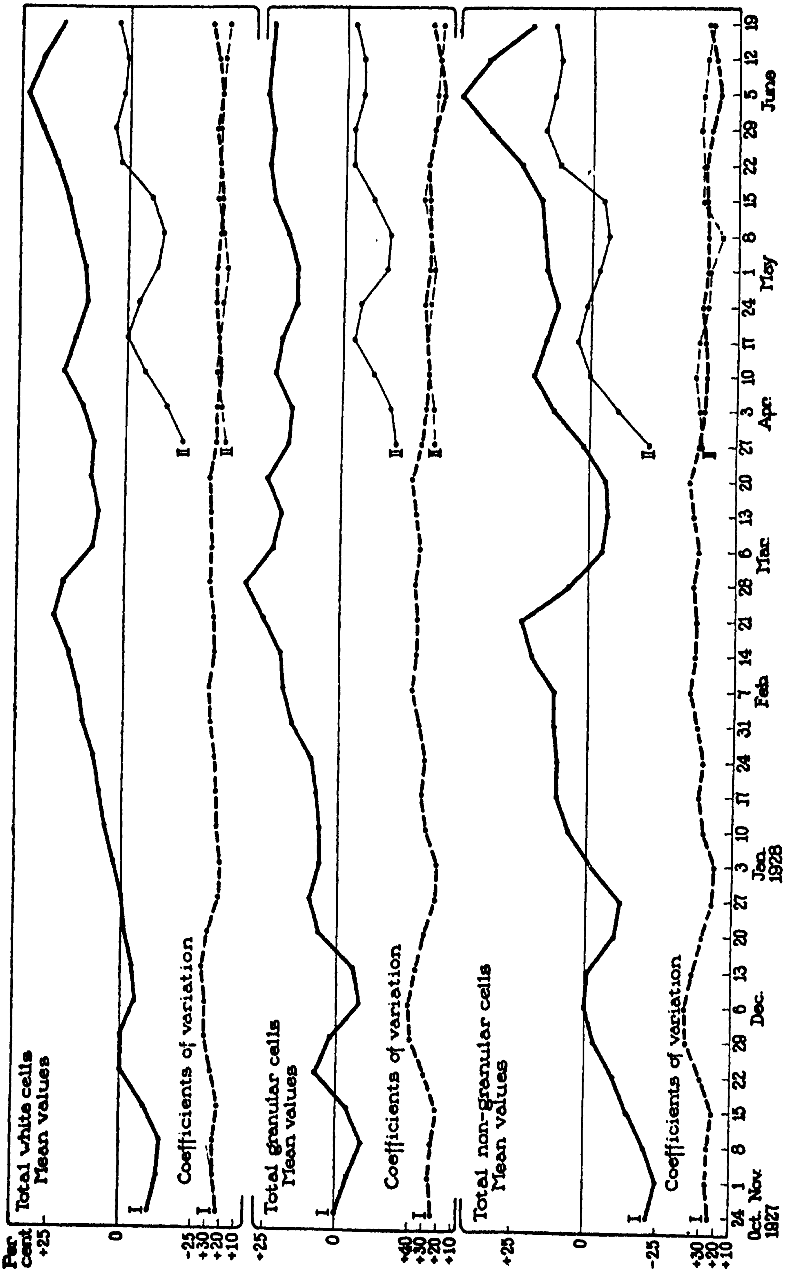
May 7	9375 ± 259	1212	12.92	6066 ± 215	1010	16.64	3273 ± 133	732	22.37
May 14	8067 ± 342	1523	18.87	4901 ± 232	1086	22.14	3136 ± 134	739	23.57
May 21	7478 ± 275	1223	16.35	5241 ± 236	1106	21.11	2237 ± 81	490	21.89
May 28	8439 ± 351	1560	18.48	5421 ± 273	1278	23.67	3002 ± 127	707	23.54
June 4	7183 ± 313	1392	19.38	4545 ± 269	1260	27.71	2638 ± 134	739	28.00
June 11	7006 ± 350	1557	22.22	4577 ± 237	1111	24.27	2429 ± 96	562	23.12
June 18	7031 ± 265	1113	15 83	3883 ± 162	758	19.53	3139 ± 192	1011	32.20
Mean	7996 ± 148	1183	14.79	5098 ± 130	1039	20.38	2883 ± 53	411	14.30
Minimum	6125			3595			2019		
Maximum	10185			7050			3887		

TABLE V

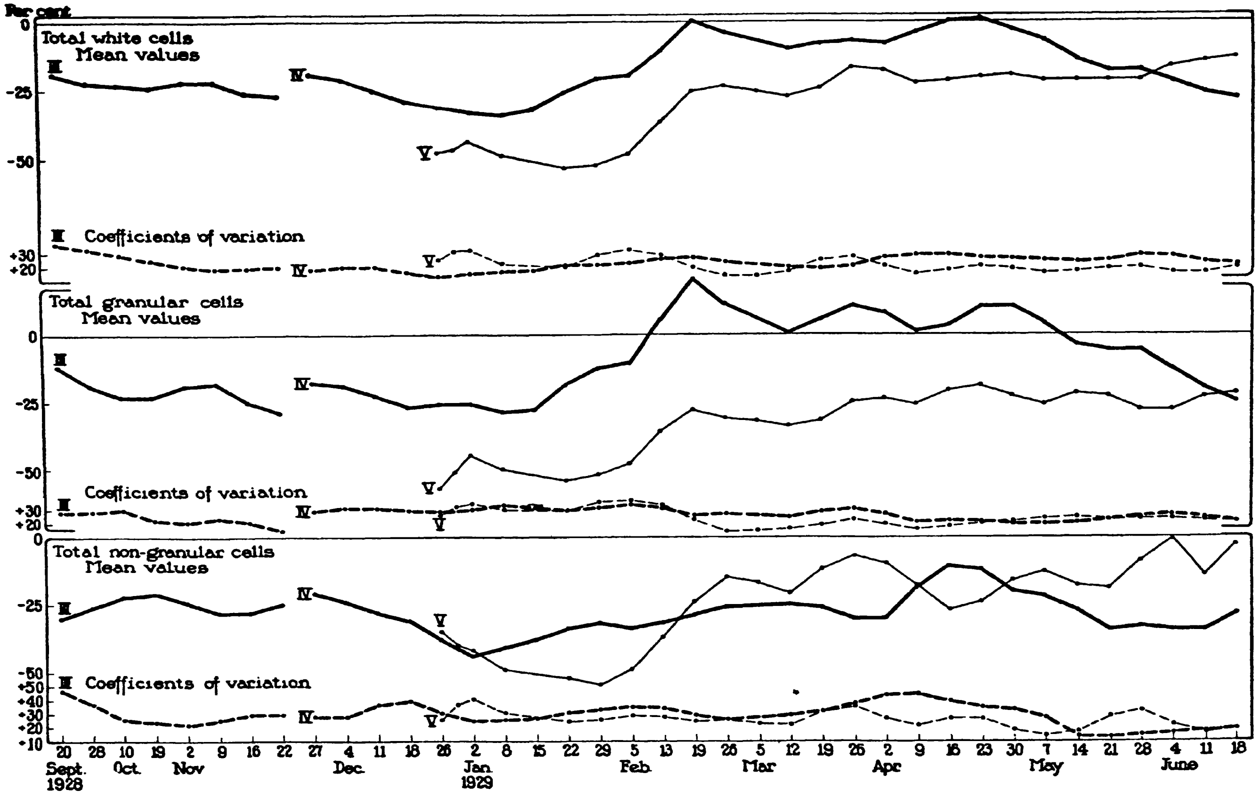
Group V—5 Rabbits. Consecutive Values for Total White Blood Cells and for Total Granular and Non-Granular Cells

Date	Total white blood cells			Granular: Neutrophiles, basophiles, and eosinophiles			Non-granular: Lymphocytes and monocytes		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
1928–29	*per cmm.*	*per cmm.*	*per cent*	*per cmm.*	*per cmm.*	*per cent*	*per cmm.*	*per cmm.*	*per cent*
Dec. 29	5310 ± 338	1119	21.07	2369 ± 178	591	24.95	2941 ± 177	586	19.93
Dec. 31	4410 ± 441	1463	33.17	2390 ± 246	817	34.18	2020 ± 222	736	36.44
Jan. 3	6260 ± 714	2367	37.81	3555 ± 371	1231	34.63	2697 ± 421	1396	51.76
Jan. 10	4510 ± 248	822	18.23	2514 ± 255	844	33.57	1996 ± 121	402	20.14
Jan. 24	4110 ± 138	458	11.14	2431 ± 131	433	17.81	1679 ± 150	496	29.54
Jan. 31	4820 ± 549	1820	37.76	2812 ± 404	1340	47.65	2008 ± 125	414	20.62
Feb. 7	4240 ± 322	1066	25.14	2451 ± 186	617	25.17	1789 ± 161	532	29.74
Feb. 15	6400 ± 757	2509	39.20	3648 ± 506	1677	45.97	2752 ± 271	898	32.63
Feb 21	7260 ± 219	725	9.99	4294 ± 200	662	15.42	2966 ± 122	405	13.65
Mar. 1	7210 ± 347	1149	15.94	3547 ± 115	380	10.71	3663 ± 390	1294	35.33
Mar. 8	7380 ± 203	672	9.11	3921 ± 168	556	14.18	3459 ± 179	593	17.14
Mar. 15	6510 ± 335	1111	17.07	3562 ± 163	540	15.16	2948 ± 171	567	19.23
Mar. 22	7010 ± 459	1521	21.70	3594 ± 166	550	15.30	3426 ± 311	1031	30.09
Mar. 29	8320 ± 855	2833	34.05	4323 ± 361	1195	27.64	3997 ± 522	1730	43.28
Apr. 5	8070 ± 430	1425	17.66	4360 ± 238	789	18.10	3710 ± 262	867	23.37
Apr. 12	6960 ± 209	692	9.94	3657 ± 138	456	12.47	3303 ± 138	456	13.81
Apr. 19	7600 ± 386	1280	16.84	4734 ± 257	852	18.00	2866 ± 271	898	31.33
Apr. 26	7220 ± 522	1730	23.96	4341 ± 281	932	21.47	2879 ± 255	845	29.35
May 3	8070 ± 401	1328	16.46	4355 ± 255	844	19.38	3715 ± 172	569	15.32
May 10	7100 ± 327	1085	15.28	3837 ± 274	908	23.66	3263 ± 127	422	12.93
May 17	7760 ± 402	1333	17.18	4350 ± 339	1123	25.82	3410 ± 159	528	15.48
May 24	7130 ± 317	1052	14.75	4364 ± 275	913	20.92	2767 ± 217	718	25.95

May 31	7990 ± 657	2179	27.27	3865 ± 265	877	22.69	4125 ± 570	1888	45.77
June 7	7510 ± 150	497	6.62	3717 ± 271	897	24.13	3793 ± 144	477	12.58
June 14	8560 ± 461	1528	17.85	4624 ± 297	986	21.32	3936 ± 194	642	16.31
June 21	8060 ± 460	1526	18.93	4095 ± 264	876	21.39	3965 ± 237	785	19.80
Mean	6761 ± 177	1334	19.73	3681 ± 97	730	19.83	3087 ± 95	718	23.26
Minimum	4110			2369			1679		
Maximum	8560			4734			3997		



TEXT-FIG. 1. Mean values for consecutive total white blood, granular and non-granular cell determinations as percentage deviations from standard values. 1927–28.



TEXT-FIG. 2. Mean values for consecutive total white blood, granular and non-granular cell determinations as percentage deviations from standard values. 1928–29.

in Text-figs. 1 and 2. The results have further been considered from the standpoint of the total granular and the total non-granular cells (Tables I to V; Text-figs. 1 and 2). In all cases, the observations are presented in the form of weekly group means together with the probable errors of the means, the standard deviations, and the coefficients of variation. The series of curves in Text-figs. 1 and 2 representing the smoothed means are drawn in the form of the percentage deviations of these values from standard values (1, 2). The standard values used are as follows: Total white cells, 9560 per cubic millimeter; total granular cells, 5505 per cubic millimeter; total non-granular cells, 4050 per cubic millimeter. Other curves illustrate the smoothed values of the coefficients of variation of the group means.

DISCUSSION AND SUMMARY

In discussing the results on the consecutive total white cell counts of 5 groups of normal rabbits studied over long periods of time, it will be convenient to consider the observations of each group in chronological order. The results will then be taken up from the standpoint of the granular and the non-granular cells.

Group I was examined from October 24, 1927 to June 19, 1928. During the 9 months of the experiment, it was found that the mean total white cell counts were considerably increased as determined by weekly observations (Table I). The character and extent of this change is shown in the curve (Text-fig. 1) which represents the percentage deviations of the smoothed weekly means from a standard value of 9560 cells per cubic millimeter.

From an initial level of 10 per cent below the standard value in October and November, 1927, there was a fairly regular increase to 25 per cent above it by the end of February; in March and April, somewhat lower levels were observed, but in May there was another rise to 35 per cent, followed in June by slightly lower counts. It should be noted that the curve of the smoothed coefficients of variation of the means (Text-fig. 1) shows only slight irregularities throughout its course, thus indicating that the alterations of the mean values were generally characteristic of the group as a whole.

In the case of Group II (Table II) which was examined from March 29 to June 19, 1928, a similar increase of the total white cells was observed.

The level of mean values was lower than that of Group I for the same period, but the general character of the increase resembled in a striking manner that of the former group (Text-fig. 1). The curve of the smoothed coefficients of variation of the weekly means of Group II is quite uniform and occupies the same level as that of Group I.

The mean white cell values of Group III, followed from September 20 to November 22, 1928 (Table III) were lower than those of Group I or of Group II and in addition, differed from these first groups in showing only minor fluctuations.

The curve illustrating these findings (Text-fig. 2) presents few irregularities and is, in fact, almost a straight line at the 20 to 25 per cent level below the standard value. The first part of the curve of the smoothed coefficients of variation of the weekly means (Text-fig. 2) is slightly higher than those of Groups I and II but the second half is entirely comparable.

The fourth group of rabbits was followed from November 27, 1928 to June 18, 1929 (Table IV). In general, the level of the white cell means (Text-fig. 2) was similar to that of Group III and lower than that of Group I examined the previous year. It was found, as was the case with Groups I and II, that these values became considerably increased.

In November and December, the lowest counts of the group were observed; in January and February, higher values prevailed; during March and April, slightly lower levels were fairly well maintained with a second increase at the end of April which in turn, was followed by a steady decline to values comparable to those observed at the beginning of the experiment. The coefficients of variation of the white cell means of Group IV were of the same general order of magnitude as those of the first 3 groups, but they were somewhat more irregular as is brought out by a comparison of the respective curves of the smoothed values shown in Text-figs. 1 and 2.

In comparing these changes with those of Group I observed during a similar period of the previous year (Text-figs. 1 and 2), it will be noted that as far as form and direction are concerned, the curves for these groups bear a general resemblance to each other up to May and June. In the case of Group I, higher levels continued to be maintained during these months although the final direction of the curve is downward; in the case of Group IV, the downward trend which began in April was continued throughout June.

The observations on Group V extended from December 19, 1928 to June 21, 1929 (Table V); this series comprised 5 as contrasted with 10 rabbits in each of the other groups. The level of the white cell means was consistently lower than that of any other group, but as was found in Groups I, II, and IV, the values became increased.

As far as trends are concerned, the first half of the Group V curve, representing the observations of December, January, February, and March, shows a striking similarity to the curve of Group IV for the same period; the latter part, however, differs in that the March level continues to be fairly constantly maintained to the end of the experiment while the curve of Group IV first rises (April) and then falls (May and June), so that eventually the two curves cross each other. The coefficients of variation of the weekly means of Group V (Text-fig. 2) are, as was the case with Group IV, more irregular than those of Groups I, II, and III, but they are of the same general order of magnitude as the others.

Turning now to a consideration of the white cells from the standpoint of their division into granular leucocytes and non-granular lymphocytes and monocytes (Table I), it will be seen by referring to Text-fig. 1, that in the case of Group I, both the granular and non-granular cells participated in the increase of the mean total white count. As far as general contour is concerned, the curves representing the means of these cells are similar to the curve of the total white cell means, but they are more irregular and their fluctuations are more pronounced, especially in the case of the non-granular cells. For the most part, the shifts in the trends of these curves are similar in direction, and not infrequently, a change in the non-granular curve precedes that of the granular curve. At the time of the pronounced drop in the total white cell means in March which occurred after a prolonged increase in values, it will be noted that there was a marked decrease in the non-granular means which was relatively much greater than the decrease of the granular cell means. In like manner, the participation of the non-granular cells in the prolonged rise of the total white cell curve during May and June was relatively much more pronounced than that of the granular leucocytes. With respect to the coefficients of variation of these means, the curves illustrating their smoothed values also show more irregularities than that of the total white means but all are of the same general order of magnitude.

In the case of Group II (Table II) the granular and non-granular cell means were found to have the same general relationship to each other and to the total white cell means as in Group I, as is illustrated by the curves in Text-fig. 1. And the coefficients of variations of these means were likewise similar to those of Group I (Table II, Text-fig. 1).

The observations on Group III (Table III) differ from those of Groups I and II in that the trends of the slight variations of the granular and non-granular means are consistently in opposite directions as is shown by the curves of Text-fig. 2. The curve representing the total white cell means is practically a straight line. The curves of the smoothed coefficients of variation for the granular and non-granular means show no significant differences from those of Groups I and II.

In the case of Group IV (Table IV), the general character of the trends of the granular and non-granular cell means with respect to each other and to the total white cell means resembled those of Groups I and II (Text-fig. 2) but in certain features, the results were different.

Thus, there are more irregularities in the curve representing the granular cells than in the curve of the non-granular cells and the fluctuations are more pronounced. Furthermore, the increase in the total mean counts during January and February and the decreasing values in May and June were participated in by a greater relative increase and decrease of the granular than of the non-granular means. The coefficients of variation of both granular and non-granular means of Group IV (Table IV, Text-fig. 2) were of the same order of magnitude as those of the previous groups.

The results of Group V (Table V) with respect to the consecutive granular and non-granular cell means resemble on the whole those of Groups I and II rather than those of Group IV, as may be seen by comparing the curves in Text-figs. 1 and 2.

While the general trend of both the granular and non-granular curves is definitely upward, the rise of the non-granular curve is more pronounced than that of the granular, indicating a greater relative increase in the mean number of non-granular cells. This feature was found in Groups I and II but the reverse occurred in Group IV. In addition, the non-granular cell curve of Group V is somewhat more irregular and the fluctuations are more marked as was also the case with Groups I and II but not with Group IV. The coefficients of variations of the granular and non-granular cell means of Group V (Table V) are of the same order of magnitude as those of the other groups (Text-fig. 2).

It should be specially noted that in the groups observed for the longest periods, the mean values of the total white cells became considerably increased. In Groups I, II, and V, followed 35, 13, and 26 weeks respectively, the values during the latter part of the experiments were all higher than those of the first weeks. Both the granular and non-granular cell means participated in this change, but from a relative standpoint, the latter values showed the greater alteration. In the case of Group IV, followed 29 weeks, a similar increase of the total white cells occurred although it was not sustained during the last 8 weeks; the granular cell means of this group showed a greater relative change than those of the non-granular cells.

In connection with these findings of augmented white cell counts over periods of several months, it might be thought that the increasing age of the rabbits was a factor of some significance; at the beginning of these experiments, the animals were considered to be 6 to 8 months old. According to the recent observations of Cheng (5), however, the numbers of leucocytes in the blood stream of rabbits 9 to 16 months of age, although fluctuating at a high level, are not increased. The question of intercurrent infections such as snuffles, as a factor in the production of increased white counts will be considered in connection with the results on the individual classes of white cells (3, 4).

The significance to be attached to the major changes in the levels of the total white, the granular, and the non-granular cell means over periods of time is indicated by the following examples of the ratios of the differences of various mean values to their probable errors.

Group Number	Total white cells			Granular cells			Non-granular cells		
	Dates of mean values		Ratio	Dates of mean values		Ratio	Dates of mean values		Ratio
I	Oct. 24	June 19	2.85	Oct. 24	June 19	2.47	Oct. 24	June 19	2.64
	Mar. 13	June 5	4.36	Dec. 6	Feb. 28	3.30	Feb. 21	Mar. 20	7.52
II	Mar. 29	June 19	7.01	Mar. 29	June 19	3.67	Mar. 29	June 19	6.61
III	Sept. 20	Nov. 16	2.00	Sept. 20	Nov. 22	3.60	Sept. 20	June 19	1.64
IV	Nov. 27	Jan. 2	4.55	Nov. 27	Feb. 19	8.33	Nov. 27	Jan. 2	5.20
	Apr. 9	June 18	5.39	Feb. 19	June 18	8.75	Jan. 2	Apr. 23	7.13
V	Dec. 29	June 21	4.82	Dec. 29	June 21	5.43	Dec. 29	Jan. 24	5.44
	Jan. 24	Apr. 5	8.76	Jan. 4	Apr. 5	7.09	Jan. 24	June 7	10.16

As was the case with the erythrocyte and hemoglobin mean values of these experiments (1), the general level of the total white cell means was higher in the groups of rabbits examined during 1927–28 than in those examined in 1928–29. This was also true of both the granular and the non-granular cells but was less pronounced in the former than in the latter class.

The present results are analogous to those of the erythrocytes and hemoglobin (1) in still another respect. It has been shown that as far as general direction or trend of the total white cell means is concerned and in the case of many individual fluctuations as well, two groups of rabbits examined during the same months behave in a similar manner. In the case of the granular and non-granular divisions of white cells, however, the resemblance is not always as striking. With the granular and non-granular cells of Groups I and II and with the granular cells of Groups IV and V, the similarity was quite pronounced, but with the non-granular cells of Groups IV and V, the parallelism was less marked.

In most cases, an alteration in the trend of the granular cell means of a group was accompanied by a movement of the non-granular cell values in the same direction but in certain instances, a change in the opposite direction was observed.

The period of most frequent point to point fluctuation of the total white cell and of the granular and non-granular cell means of the 4 groups examined for several months occurred in February, March, and April of both years (1928 and 1929). A similar but less pronounced phase of irregularity affecting chiefly the total white and the granular cells was observed in October and November of 1927 but not in the fall months of 1928.

CONCLUSIONS

Consecutive weekly observations on the total white cell count of the peripheral blood were made on 5 groups of normal rabbits, a total of 45 animals, during a period of 20 months from October, 1927 to July, 1929. The duration of individual group examinations varied from 8 to 35 weeks.

In the case of 4 groups followed 13 to 35 weeks, the general trend of the total white cell means was toward increasing values; with the group followed 8 weeks, the means were maintained at a constant level.

The changes in the levels of the granular cell means were usually accompanied by changes in a similar direction of the non-granular cell means. In the case of 3 of the 4 groups followed for the longest periods, the greatest relative alterations occurred in the non-granular cells.

The fluctuations in the mean values of the total white cells and of the granular and the non-granular cells which were observed in one group of rabbits, were also generally observed in another group examined during the same months.

The period of greatest irregularity in the mean values of the total white cell means and of the granular and the non-granular cell means as well, occurred during the late winter and spring months of both years.

The general level of the total white cell mean values in the groups examined during 1927–28 was higher than that of the groups observed during 1928–29. A similar difference was found in the granular and non-granular mean values but it was somewhat less marked in the case of the granular cells.

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STUDIES IN THE BLOOD CYTOLOGY OF THE RABBIT

IV. CONSECUTIVE NEUTROPHILE, BASOPHILE, AND EOSINOPHILE OBSERVATIONS ON GROUPS OF NORMAL RABBITS

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The two preceding papers of this series have dealt with the consecutive observations on the erythrocytes and the hemoglobin content (1) and with the total white cells (2) of the peripheral blood of normal male rabbits in a series of experiments extending over long periods of time. In the present paper, the results pertaining to the neutrophile (pseudo-eosinophile), basophile, and eosinophile cells in the same five groups of animals are reported while in the succeeding paper (3), the lymphocytes and monocytes will be considered. Since this study was primarily undertaken for the purpose of obtaining information on the character of the general trends of cell levels over prolonged periods, the results are at present considered from the group rather than from the individual animal standpoint and the findings have been analyzed upon the basis of consecutive mean values obtained at weekly intervals.

Materials and Methods

These subjects have been fully discussed in connection with the erythrocyte and hemoglobin results (1) and need only be referred to here. The first four groups of rabbits comprised 10 animals each and the fifth group, 5 animals. The period of observation extended from October, 1927 to July, 1929 and the following numbers of weekly examinations were made: Group I, 35; Group II, 13; Group III, 8; Group IV, 29; Group V, 26. For the differential white cell counts, the supravital neutral red technic was used and 100 cells were counted in each specimen.

RESULTS

The results of these experiments dealing with the neutrophile, basophile, and eosinophile cell content of the peripheral blood of normal

TABLE I

Group I—10 Rabbits. Consecutive Values for Neutrophiles, Basophiles, and Eosinophiles

Date	Neutrophiles			Basophiles			Eosinophiles		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
1927–28	*per cmm.*	*per cmm.*	*per cent*	*per cmm.*	*per cmm.*	*per cent*	*per cmm.*	*per cmm.*	*per cent*
Oct. 24*..................	4038 ± 279	1307	32.37	1276 ± 156	730	57.21	273 ± 43	201	73.63
Nov. 1..................	4340 ± 295	1383	31.87	791 ± 48	225	28.45	162 ± 12	56	34.57
Nov. 8**..................	3775 ± 256	1198	31.74	902 ± 85	398	44.12	271 ± 28	131	48.34
Nov. 15..................	3266 ± 173	811	24.83	1235 ± 128	602	48.74	400 ± 80	376	94.00
Nov. 22..................	5001 ± 388	1820	36.39	1045 ± 122	574	54.93	476 ± 87	408	85.71
Nov. 29..................	4470 ± 638	2992	66.94	813 ± 112	525	64.58	211 ± 31	143	67.77
Dec. 6..................	3877 ± 394	1848	47.67	842 ± 75	350	41.57	268 ± 29	135	50.37
Dec. 13..................	3774 ± 355	1663	44.06	774 ± 95	447	57.75	234 ± 40	185	79.06
Dec. 20..................	4905 ± 413	1934	39.43	1024 ± 99	465	45.41	322 ± 32	149	46.27
Dec. 27..................	4818 ± 265	1243	25.80	963 ± 124	579	60.12	202 ± 33	154	76.24
Jan. 3..................	4618 ± 253	1185	25.66	905 ± 72	336	37.13	288 ± 44	207	71.88
Jan. 10..................	4488 ± 365	1712	38.15	1138 ± 129	606	53.25	201 ± 30	142	70.65
Jan. 17..................	4566 ± 459	2151	47.11	1180 ± 161	755	63.98	154 ± 30	140	90.91
Jan. 24..................	4242 ± 294	1380	32.53	1373 ± 168	787	57.32	277 ± 52	244	88.09
Jan. 31..................	5725 ± 529	2481	43.34	6686 ± 157	738	107.58	180 ± 28	130	72.22
Feb. 7..................	4820 ± 472	2215	45.95	1358 ± 177	831	61.19	299 ± 40	189	63.21
Feb. 14..................	5688 ± 530	2485	43.69	763 ± 65	305	39.97	193 ± 37	173	89.64
Feb. 21..................	4544 ± 366	1715	37.74	1710 ± 113	529	30.94	290 ± 42	197	67.93
Feb. 28..................	6687 ± 883	4142	61.94	1111 ± 157	735	66.16	244 ± 46	215	88.11
Mar. 6..................	4790 ± 289	1356	28.31	1291 ± 233	1092	84.59	308 ± 45	210	68.18
Mar. 13..................	5208 ± 459	2151	41.30	787 ± 94	443	56.29	191 ± 40	186	97.38
Mar. 20..................	6653 ± 816	3826	57.51	707 ± 75	353	49.93	255 ± 65	305	119.61

Mar. 27	4662 ± 375	1756	37.67	1231 ± 115	538	43.70	170 ± 33	153	90.00
Apr. 3	4851 ± 367	1719	35.44	1046 ± 108	506	48.37	306 ± 33	154	50.33
Apr. 10	5793 ± 401	1880	32.45	1078 ± 127	597	55.38	400 ± 58	274	68.50
Apr. 17	5060 ± 359	1684	33.28	1108 ± 124	579	52.26	217 ± 23	109	50.23
Apr. 24	5256 ± 516	2419	46.02	1046 ± 92	429	41.01	220 ± 57	268	121.82
May 1	4851 ± 278	1304	26.88	1121 ± 69	321	28.64	159 ± 39	183	115.09
May 8	5055 ± 401	1880	37 19	1399 ± 173	813	58.11	190 ± 30	139	73.16
May 15	5111 ± 382	1791	35.04	1234 ± 107	501	40.60	416 ± 89	416	85.58
May 22	5901 ± 597	2797	47.40	1103 ± 130	609	55.21	213 ± 33	155	72.77
May 29	5269 ± 382	1789	33.95	981 ± 83	389	39.65	193 ± 29	135	69.95
June 5	5751 ± 361	1691	29.40	1462 ± 127	595	40.70	233 ± 50	232	99.57
June 12	5048 ± 334	1567	31.04	1339 ± 162	760	56.76	270 ± 28	133	49.26
June 19	5343 ± 425	1992	37.28	1364 ± 183	857	62.83	268 ± 42	197	73.51
Mean	4919 ± 268	2349	47.75	1091 ± 27	238	21.81	256 ± 9	76	29.69
Minimum	3266			686			154		
Maximum	6687			1710			476		

* October 24 and 26.

** November 4 and 9.

TABLE II

Group II—10 Rabbits. Consecutive Values for Neutrophiles, Basophiles, and Eosinophiles

Date	Neutrophiles			Basophiles			Eosinophiles		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
1928	*per cmm.*	*per cmm.*	*per cent*	*per cmm.*	*per cmm.*	*per cent*	*per cmm.*	*per cmm.*	*per cent*
Mar. 29	3582 ± 284	1330	37.13	579 ± 42	197	34.02	139 ± 25	116	83.45
Apr. 3	3821 ± 220	1031	26.98	768 ± 88	411	53.52	155 ± 19	91	58.71
Apr. 10	3825 ± 340	1593	41.65	550 ± 55	258	46.91	140 ± 25	117	83.57
Apr. 17	4481 ± 320	1501	33.50	882 ± 94	442	50.11	277 ± 76	358	129.24
Apr. 24	4420 ± 403	1890	42.76	859 ± 110	520	60.54	152 ± 18	86	56.58
May 1	3123 ± 106	497	15.91	831 ± 108	505	60.77	150 ± 26	124	82.67
May 8	4111 ± 280	1311	31.89	871 ± 49	231	26.52	111 ± 21	96	86.49
May 15	3515 ± 359	1685	47.94	590 ± 65	306	51.86	180 ± 27	128	71.11
May 22	4920 ± 333	1559	31.69	954 ± 100	467	48.95	258 ± 33	155	60.08
May 29	4008 ± 350	1642	40.97	726 ± 88	411	56.61	113 ± 30	141	124.78
June 5	4348 ± 281	1319	30.34	1103 ± 84	392	35.54	118 ± 18	86	72.88
June 12	3653 ± 213	999	27.35	958 ± 113	528	55.11	111 ± 16	76	68.47
June 19	4632 ± 190	889	19.19	856 ± 109	510	59.58	175 ± 25	116	66.29
Mean	4034 ± 92	491	12.17	810 ± 29	157	19.38	160 ± 10	51	31.88
Minimum	3123			550			111		
Maximum	4920			1103			277		

TABLE III

Group III—10 Rabbits. Consecutive Values for Neutrophiles, Basophiles, and Eosinophiles

Date	Neutrophiles			Basophiles			Eosinophiles		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
1928	*per cmm.*	*per cmm.*	*per cent*	*per cmm.*	*per cmm.*	*per cent*	*per cmm.*	*per cmm.*	*per cent*
Sept. 20	4777 ± 484	2270	47.52	437 ± 42	198	45.31	144 ± 47	221	153.47
Sept. 28	3002 ± 163	762	25.13	586 ± 50	232	39.59	179 ± 30	140	78.21
Oct. 10	3913 ± 467	2188	55.93	671 ± 84	395	58.87	215 ± 32	149	69.30
Oct. 19	2682 ± 263	1235	46.05	835 ± 66	308	36.89	157 ± 18	86	54.78
Nov. 2	3853 ± 281	1315	34.13	733 ± 64	301	41.06	168 ± 39	184	109.52
Nov. 9	3620 ± 213	1000	27.16	836 ± 102	477	57.06	257 ± 47	218	84.82
Nov. 16	3004 ± 261	1223	40.71	684 ± 57	267	39.04	252 ± 34	157	62.30
Nov. 22	3006 ± 249	1167	38.44	708 ± 69	321	45.34	178 ± 25	115	64.61
Mean	3482 ± 154	647	18.58	686 ± 29	122	17.78	194 ± 10	40	20.62
Minimum	2682			437			144		
Maximum	4777			836			257		

TABLE IV

Group IV—10 Rabbits. Consecutive Values for Neutrophiles, Basophiles, and Eosinophiles

Date	Neutrophiles			Basophiles			Eosinophiles		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
1928–29	*per cmm.*	*per cmm.*	*per cent*	*per cmm.*	*per cmm.*	*per cent*	*per cmm.*	*per cmm.*	*per cent*
Nov. 27	3880 ± 246	1154	29.75	602 ± 86	401	66.67	111 ± 25	118	106.62
Dec. 4	3639 ± 286	1342	36.88	742 ± 96	450	60.60	73 ± 14	64	88.39
Dec. 11	3535 ± 280	1312	37.11	812 ± 55	257	31.59	120 ± 20	96	80.06
Dec. 18	2938 ± 158	742	25.23	668 ± 77	360	53.87	64 ± 14	64	99.56
Dec. 26	3440 ± 258	1208	35.12	670 ± 74	349	52.15	82 ± 18	86	104.89
Jan. 2	3530 ± 174	816	23.12	735 ± 63	297	40.39	55 ± 15	71	128.90
Jan. 8	2870 ± 279	1310	45.64	608 ± 63	296	48.61	117 ± 28	133	113.73
Jan. 15	3396 ± 172	808	23.78	573 ± 64	300	52.31	117 ± 17	81	69.22
Jan. 22	3221 ± 255	1195	37.11	758 ± 77	362	47.81	123 ± 21	100	81.44
Jan. 29	4442 ± 292	1371	30.87	965 ± 83	387	40.14	103 ± 20	95	92.19
Feb. 5	3481 ± 331	1551	44.55	552 ± 36	167	30.16	83 ± 18	84	100.69
Feb. 13	5170 ± 378	1771	34.27	632 ± 78	367	58.03	177 ± 29	137	77.51
Feb. 19	6085 ± 328	1536	25.24	741 ± 71	334	45.02	224 ± 61	285	127.40
Feb. 26	5403 ± 360	1687	31.21	850 ± 127	594	69.87	112 ± 22	101	90.06
Mar. 12	4251 ± 215	1010	23.75	408 ± 53	247	60.53	72 ± 16	77	107.43
Mar. 19	5544 ± 426	1996	36.00	514 ± 30	142	27.52	201 ± 30	139	69.35
Mar. 26	5483 ± 351	1644	29.97	400 ± 44	204	51.00	44 ± 10	47	105.90
Apr. 2	5342 ± 384	1799	33.67	612 ± 58	273	44.53	105 ± 25	115	109.60
Apr. 9	5160 ± 133	625	12.11	377 ± 31	147	39.08	149 ± 26	124	83.08
Apr. 16	5396 ± 314	1470	27.24	434 ± 56	262	60.36	55 ± 16	76	137.40
Apr. 23	5609 ± 218	1023	18.23	505 ± 57	267	52.90	132 ± 39	185	140.10
Apr. 30	5384 ± 220	1031	19.14	474 ± 66	311	65.54	54 ± 15	70	128.90

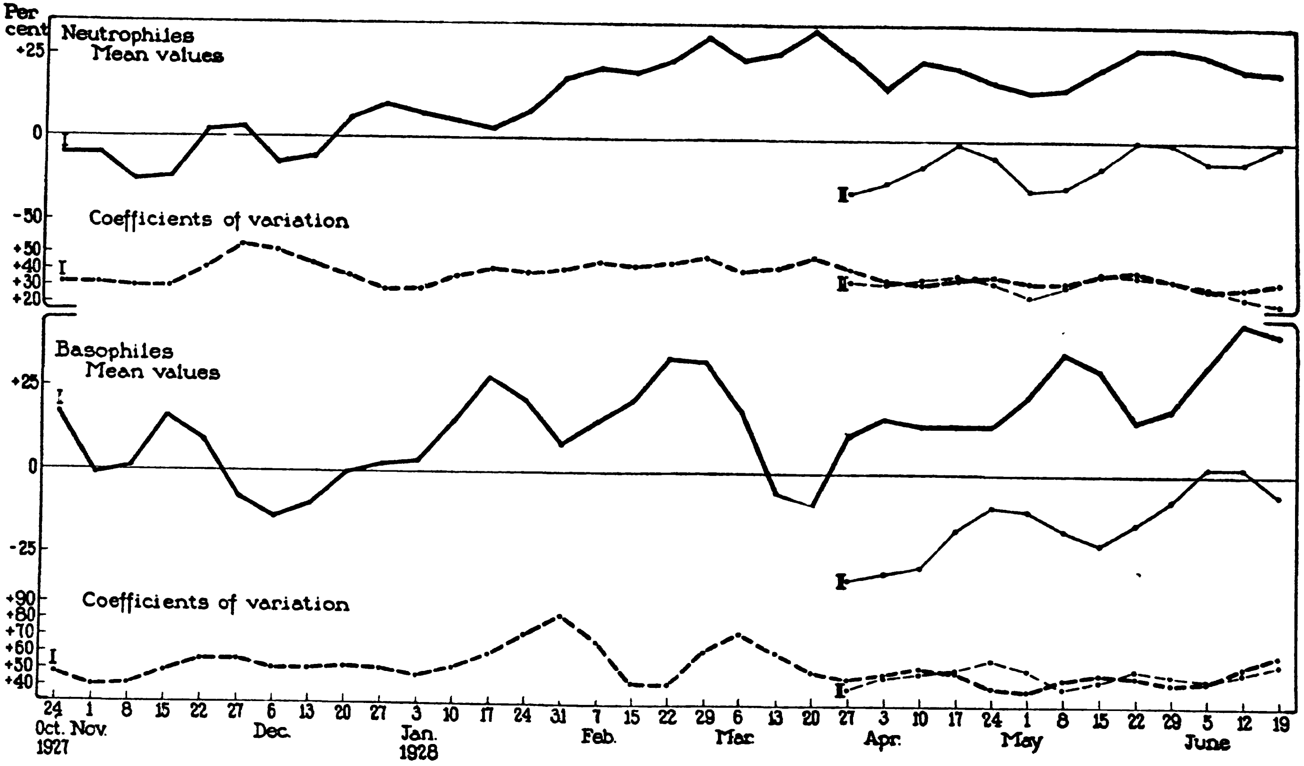
May 7	5434 ± 332	1557	28.66	479 ± 60	280	58.41	153 ± 28	130	84.64
May 14	4380 ± 199	883	20.15	490 ± 104	464	94.59	106 ± 8	35	117.63
May 21	4826 ± 248	1101	22.80	388 ± 75	332	85.47	26 ± 12	55	210.00
May 28	4769 ± 231	1026	21.51	564 ± 100	443	78.58	87 ± 27	122	139.65
June 4	4123 ± 263	1170	28.37	386 ± 52	231	59.85	36 ± 13	56	155.27
June 11	4085 ± 209	930	22.77	455 ± 68	301	66.04	37 ± 9	42	114.59
June 18	3464 ± 164	688	19.86	343 ± 50	211	61.57	76 ± 17	71	93.00
Mean	4423 ± 117	933	21.09	577 ± 20	157	27.20	100 ± 6	48	48.01
Minimum	2870			343			26		
Maximum	6085			965			224		

TABLE V

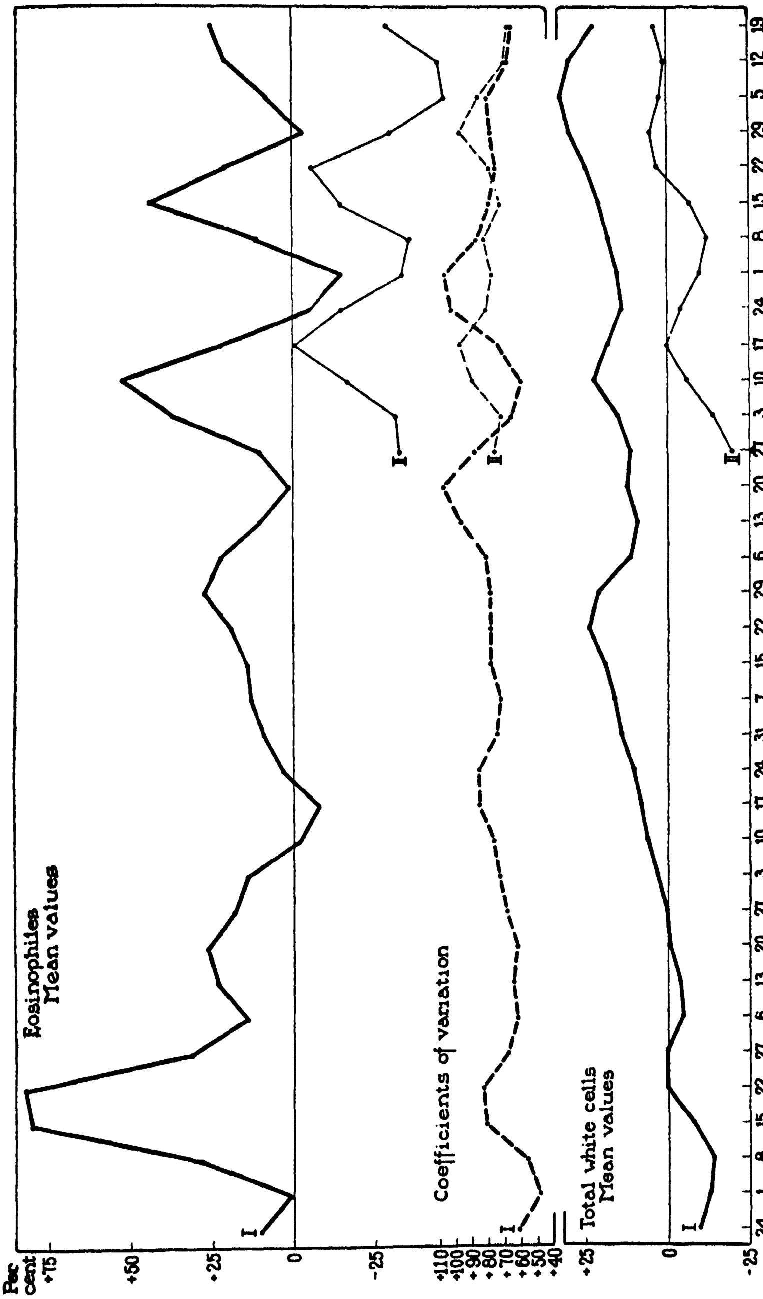
Group V—5 Rabbits. Consecutive Values for Neutrophiles, Basophiles, and Eosinophiles

Date	Neutrophiles			Basophiles			Eosinophiles		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
1928–29	*per cmm.*	*per cmm.*	*per cent*	*per cmm.*	*per cmm.*	*per cent*	*per cmm.*	*per cmm.*	*per cent*
Dec. 21	2062 ± 191	632	30.65	290 ± 57	188	64.83	17 ± 6	21	123.53
Dec. 31	1892 ± 197	654	34.57	498 ± 66	220	44.18	19 ± 7	24	126.32
Jan. 3	2815 ± 326	1081	38.40	614 ± 41	135	21.99	117 ± 32	106	90.60
Jan. 10	1915 ± 251	833	43.50	525 ± 46	151	28.76	74 ± 15	51	68.92
Jan. 24	2014 ± 115	382	18.97	381 ± 38	127	33.33	36 ± 13	44	122.22
Jan. 31	2097 ± 272	902	43.01	642 ± 171	568	88.47	73 ± 13	42	57.53
Feb. 7	1994 ± 162	537	26.97	333 ± 59	196	58.86	124 ± 39	130	104.84
Feb. 15	2999 ± 392	1298	43.28	529 ± 92	304	57.47	121 ± 26	85	70.25
Feb. 21	3677 ± 176	584	15.88	461 ± 29	96	20.82	156 ± 35	117	75.00
Mar. 1	2850 ± 84	279	9.79	507 ± 88	292	57.59	190 ± 39	130	68.42
Mar. 8	3320 ± 145	480	14.46	454 ± 20	65	14.32	147 ± 21	68	46.26
Mar. 15	2680 ± 135	449	16.75	656 ± 46	224	34.15	226 ± 53	175	77.43
Mar. 22	3012 ± 140	465	15.44	363 ± 61	202	55.65	219 ± 38	127	57.99
Mar. 29	3650 ± 283	937	25.67	484 ± 118	390	80.58	189 ± 41	136	71.96
Apr. 5	3669 ± 207	685	18.67	512 ± 43	144	28.13	180 ± 20	67	37.22
Apr. 12	3094 ± 186	617	19.94	382 ± 62	204	53.40	181 ± 35	115	63.54
Apr. 19	4171 ± 246	817	19.59	410 ± 32	106	25.85	153 ± 36	118	77.12
Apr. 26	3715 ± 213	705	18.98	528 ± 76	253	47.92	98 ± 24	80	81.63
May 3	3612 ± 294	975	26.99	615 ± 57	190	30.89	127 ± 39	130	102.36
May 10	3171 ± 240	797	25.13	458 ± 64	211	46.07	208 ± 67	221	106.25
May 17	3774 ± 322	1068	28.30	461 ± 40	132	28.63	115 ± 29	95	82.61

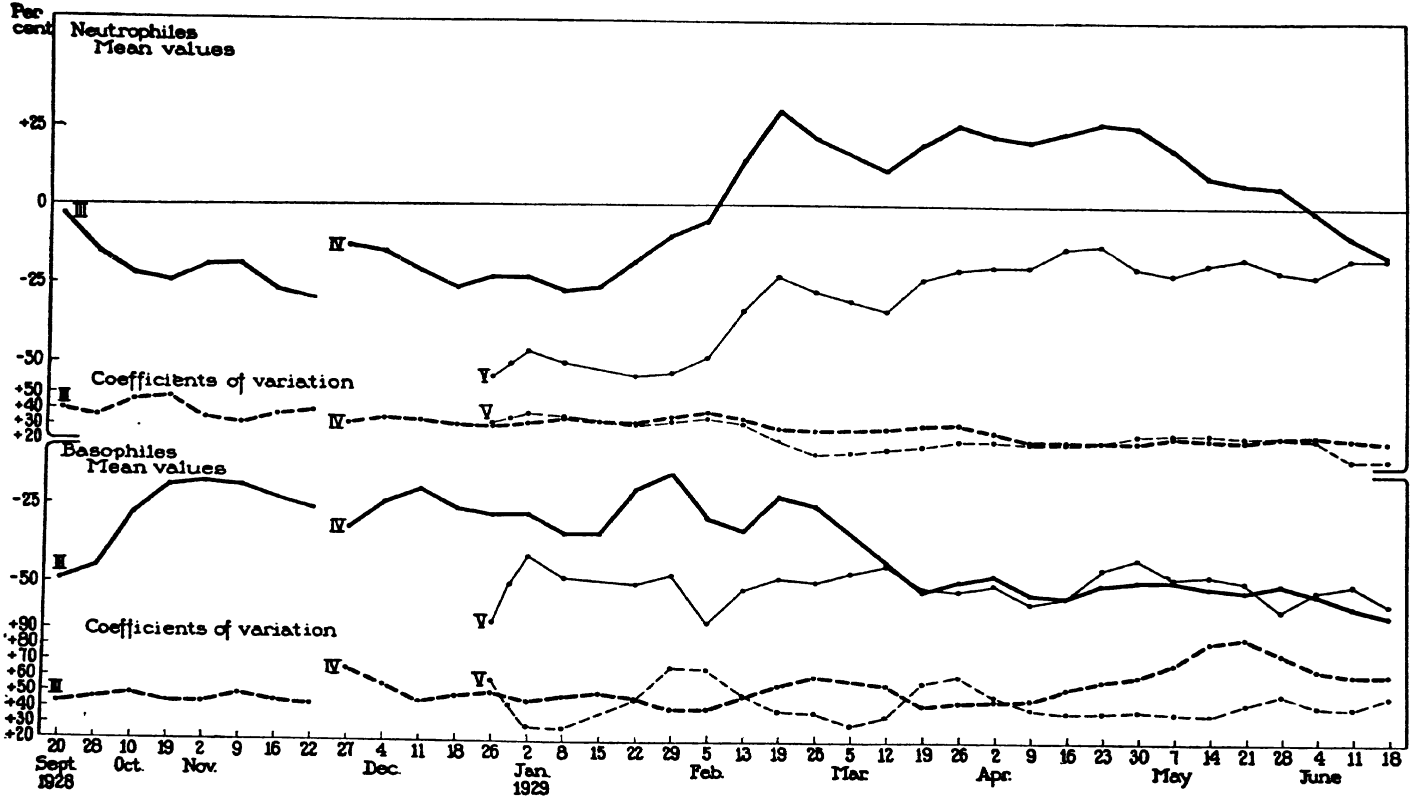
May 24	3604 ± 239	791	21.95	634 ± 86	284	44.79	126 ± 16	54	42.86
May 31	3616 ± 276	916	25.33	212 ± 36	120	56.60	37 ± 14	47	12.70
June 7	3045 ± 215	714	23.45	546 ± 66	220	40 29	126 ± 35	115	91.27
June 14	4005 ± 227	752	18.78	492 ± 54	178	36.18	127 ± 41	135	106.30
June 21	3519 ± 211	698	19.84	382 ± 65	214	56.02	194 ± 50	164	84.54
Mean	3076 ± 92	692	22.50	476 ± 14	109	22.90	130 ± 8	59	45.38
Minimum	1892			212			17		
Maximum	4171			652			226		



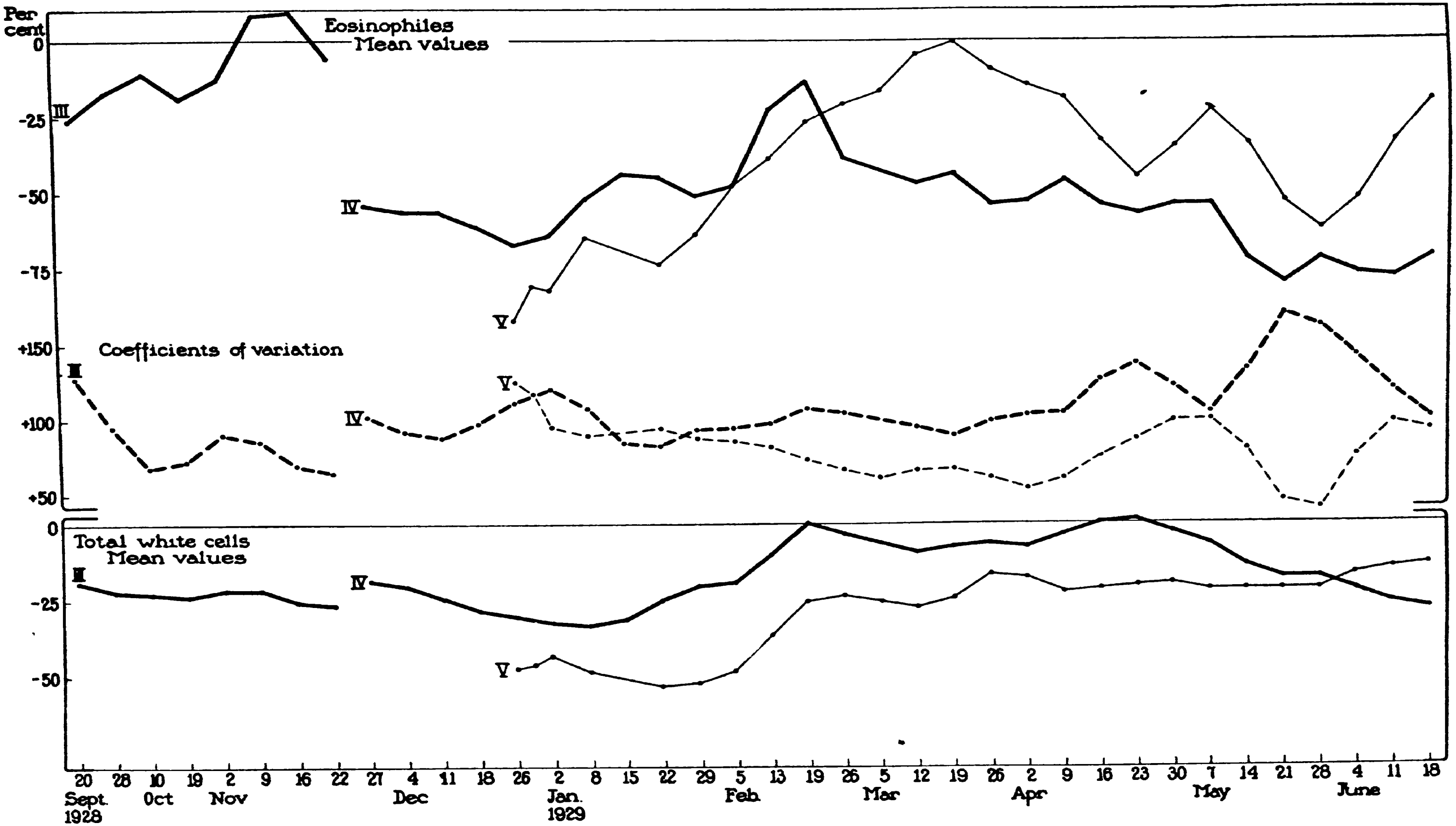
TEXT-FIG. 1. Mean values for consecutive neutrophile and basophile determinations as percentage deviations from standard values. 1927–28.



TEXT-FIG. 2. Mean values for consecutive eosinophile and total white blood cell determinations as percentage deviations from standard values. 1927–28.



TEXT-FIG. 3. Mean values for consecutive neutrophile and basophile determinations as percentage deviations from standard values. 1928–29.



Text-Fig. 4. Mean values for consecutive eosinophile and total white blood cell determinations as percentage deviations from standard values. 1928–29.

rabbits examined over long periods of time are presented in the form of consecutive weekly mean values for each group of animals (Tables I to V). In addition, the probable errors of the means, the standard deviations, and the coefficients of variation are given. The series of curves in Text-figs. 1 to 4 representing the mean values, are drawn from the percentage deviations of the smoothed means from the following standard values (1, 4): neutrophiles, 4340 cells per cubic millimeter; basophiles, 950 cells per cubic millimeter; eosinophiles, 215 cells per cubic millimeter. The curves of the smoothed coefficients of variation of the means are also given in Text-figs. 1 to 4. These charts also include curves which represent the total white cell means in terms of the percentage deviations of the smoothed means from a standard value of 9560 cells per cubic millimeter, in order that comparisons may readily be made between the results on the various granular leucocytes and those on the total white count without referring to the preceding paper (2) in which the latter observations are discussed.

DISCUSSION AND SUMMARY

For convenience of discussion, the results of these experiments dealing with consecutive neutrophile, basophile, and eosinophile counts will be considered in the chronological order of the five groups of rabbits studied.

Group I was examined from October 24, 1927 to June 21, 1928 (Table I). It will be seen by referring to Text-fig. 1, that the mean numbers of neutrophiles had increased approximately 30 per cent by the second half of the experiment and that thereafter, this higher level was generally maintained. In October, November, and December, the general level of the curve fluctuates slightly below and above the standard value of 4340 cells per cubic millimeter represented by the base line. During January, February, and March, a decided upward trend is noted and thereafter to the last of June, a slightly lower level is observed.

The mean numbers of basophiles in Group I (Table I) were also increased during the experiment, but as will be seen from the curve in Text-fig. 1, the change was accomplished in a much more irregular fashion than in the case of the neutrophiles. During October and November, the general trend of the curve is downward from an initial level above the base line (950 cells per cubic millimeter); in December, January, and February, the curve describes an irregular but marked rise which is followed by an abrupt fall to the November level; at the end of March and continuing through April, May, and June, there is a second rise which carries the curve to the highest level observed (55 per cent above the base line). During these periods of increasing value, there were two interruptions, the first occurring the end of January and the second in May.

In the case of the eosinophiles of Group I (Table I) pronounced variations in mean numbers were observed, as is shown by the curve in Text-fig. 2. Throughout November and again in April and May, large numbers of these cells were observed which account for the three prominent and abrupt peaks of the curve. Following each marked increase, the mean values sank to a comparatively low level, but on the whole, the means throughout the experiment were higher than the standard value of 215 cells per cubic millimeter.

Comparing the general changes in the mean values of the three classes of granular leucocytes with that of the total white cells (Text-fig. 2), it will be noted that during the first half of the experiment (October to March) increasing values were observed in the total white cells, the neutrophiles, and the basophiles (after November) which are indicated by the upward direction of their curves. On the other hand, this feature was not found in the eosinophile results until the January and February observations. During the latter part of February and throughout March, lower levels prevailed in the total white cell, the basophile, and the eosinophile means but in the case of the neutrophiles, the values although comparatively irregular, were on the whole not diminished. In the last period of the experiment, the trend of the total white cells was again upward, a movement characteristic also of the basophiles and to a slight extent of the neutrophiles. The later eosinophile means which showed no consistent trend in one direction or another were characterized by a series of abrupt and pronounced swings. The high points of these fluctuations (February 29, April 10, and May 15), however, occurred at the time of high neutrophile values and somewhat analogous findings were observed in the earlier weeks of the experiment (November 22 and December 20).

The coefficients of variation of the neutrophile means of Group I (Table I) are fairly uniform although their order of magnitude is high.

The curve of the smoothed values (Text-fig. 1) shows variations within a 10 per cent range at the general level of 30 to 40 per cent except in November and December when the abrupt alteration of mean values is accompanied by a sharp upward turn of the coefficient curve. In the case of the basophiles (Table I) the curve of the smoothed coefficients of variation (Text-fig. 1) is fairly regular at the 40 to 50 per cent level except for two major upward swings the end of January and the first of March which accompany not increased, but diminished mean values. In the last third of the experiment, during which the basophile means rose to the highest figure observed, the coefficients of variation are comparatively uniform. The coefficients of variation of the eosinophile means (Table I) are of a very high order of magnitude, the curve of the smoothed values ranging from 47 to 107 per cent. The highest points on the curve correspond to the period of extremely high mean values in November but to the low mean values in January, March, and May which immediately succeeded abruptly increased counts. During these phases of augmented mean values, the coefficients of variation are smaller so that the curve is directed downward.

Group II was examined from March 29 to June 19, 1928 (Table II). The curves illustrating the neutrophile, the basophile, and the eosinophile means as percentage deviations from standard values (Text-figs. 1 and 2) show a striking resemblance in general contour to the curves of Group I for this period as is also the case with the total white cells (Text-fig. 2); the levels of the means of Group II, however, are considerably lower. The two prominent peaks of the eosinophile curve in April and May, occurring almost at the same time as those of Group I, are worthy of special note in view of the comparatively small numbers of these cells.

The coefficients of variation of these three cell means of Group II (Table II) are of the same order of magnitude as those of Group I. The curves of the smoothed values (Text-figs. 1 and 2) are similar to those of Group I except in the case of the eosinophiles in which one of the two highest points corresponds to and the other directly follows, high mean values.

Group III was examined from September 20 to November 22, 1928 (Table III). The neutrophile, basophile, and eosinophile mean values were generally lower than those of Group I and were more comparable to those of Group II, as may be seen by comparing the respective curves in Text-figs. 1, 2, 3, and 4. In the case of the neutrophile curve, there is a prevailing downward trend which is described in a fairly regular fashion while the direction of the basophile and eosinophile curves is upward. The curve for the total white cells, on the other hand, is almost a straight line (Text-fig. 4).

The coefficients of variation of these means (Table III) are of similar orders of magnitude to those of Groups I and II, but contrary to what was found in Group I, the curve of the smoothed neutrophile values shows more irregularities than that of the basophiles (Text-fig. 3). The highest point of the eosinophile coefficient curve (Text-fig. 4) corresponds to the smallest mean value, a feature which was generally characteristic of Group I.

Group IV was examined from November 27, 1928 to June 18, 1929 (Table IV). On the whole, the mean values for the neutrophile, the basophile, and the eosinophile cells were generally smaller than those of Group I. In the case of the neutrophiles (Text-fig. 3), low initial levels prevailed until the middle of January, at which time a pronounced increase occurred and a level of approximately 25 per cent above the standard value was maintained until the end of April, after which progressively smaller values were found. In general form, the neutrophile curve resembles that of the total white cells. The highest basophile values, on the other hand, were observed in the first half of the experiment as is shown by the curve in Text-fig. 3; in the second half, the curve occupies a uniform position 25 per cent lower than its first level. The two small peaks in the basophile curve at the end of January and toward the end of February occur at the time of the pronounced upward swing of the neutrophile curve. The trend of the first part of the eosinophile curve (Text-fig. 4) is in a rising direction culminating abruptly in a sharp peak the latter part of February. From then onward to the end of the experiment, the curve describes an irregular fall and ends at a lower level than that of the initial observations.

The coefficients of variation of the neutrophile, basophile, and eosinophile means of Group IV (Table IV) are comparable in their orders of magnitude to those of the other groups. From the curves of the smoothed values, it will be seen that that of the neutrophiles was more uniform than that of the basophiles (Text-fig. 3) which is the case with Groups I and II but not with Group III. It should be noted that the marked changes in the level of the neutrophile mean values are not accompanied by pronounced fluctuations of the coefficient curve. The general level of the basophile coefficient curve is fairly well maintained until April and May when an upward swing occurs although no accompanying change in the curve representing the mean values is seen. In the case of the eosinophiles (Text-fig. 4), the coefficient curve is fairly regular until April and May when there are two abrupt upward fluctuations. These changes occur during diminishing mean values and the second high coefficient peak follows a marked drop in the mean values during the first part of May.

Group V was examined from December 29, 1928 to June 21, 1929 (Table V). The curves representing the mean values of the neutrophile, basophile, and eosinophile cells (Text-figs. 3 and 4) are similar in general outline to those of Group IV examined during the same months. In the case of the neutrophiles, however, much lower values prevailed until the end of the experiment. A comparison of the neutrophile and total white cell curves brings out the fact of their similarity with respect to general form as was also found with the other groups. The basophile curve for Group V which is relatively stable at a level of 50 per cent below the standard value shows the smallest fluctuations of any of the basophile curves. In many places, however, those which do occur correspond to similar alterations in the curve of the Group IV basophiles. In the case of the eosinophiles, the curves of the two groups bear a general resemblance to each other but the swings of the Group V curve are less pronounced, the summit of its marked rise in the middle of the observation period occurs somewhat later, and its second half occupies a higher level.

The coefficients of variation of the neutrophile means of Group V (Table V) are generally comparable to those of Group IV (Text-fig. 3); those of the basophiles (Table V) are on the whole somewhat smaller (Text-fig. 3). The coefficients of the eosinophile means of Group V are generally smaller than those of Group IV (Table IV). In the curve of the smoothed values (Text-fig. 4) it should be noted that comparatively low levels prevail during February and March in which months the mean values were progressively high. In its latter portion, the curve described two abrupt upward swings, the first of which corresponds to decreasing and the second to increasing mean values.

There are certain features of these results obtained from the observations of many weeks to which attention may now be especially directed. In the first place, the neutrophiles, as would be expected in view of the relative number of these cells, influence the total white cell count to the extent that the curve representing the consecutive smoothed mean values of the one usually resembles quite closely the curve of the other.

In the experiments carried out for the longer periods, Groups I and II in 1927–28 and Groups IV and V in 1928–29, an increase in the neutrophile mean values occurred in the late winter and early spring months but during May and June, lower or stationary levels prevailed. In Group III examined during the autumn of 1928, the trend was in the direction of smaller values, a finding which is in accord with the low values of the other groups in October, November, and December of both years. The results pertaining to the basophiles differed in the two years. In 1927–28, there was a general increase in mean values which continued through the spring months; in the second year, the trend was toward smaller values. The rising basophile values of Group III in the fall of 1928 were in accord with the early findings of Group IV, the first examination of which followed the last observations of Group III. In the case of the eosinophiles, the results were more irregular. Their most prominent feature was the abrupt and very pronounced increase and decrease of mean values occurring in November of 1927 and in the late winter and spring months of 1928 and 1929.

That the variations in the general levels of the neutrophile, the basophile, and the eosinophile mean values observed in these experiments have a statistical significance is shown by the following examples of the ratios of the differences of certain means to their probable errors:

Group number	Neutrophiles			Basophiles			Eosinophiles		
	Dates of mean values		Ratio	Dates of mean values		Ratio	Dates of mean values		Ratio
I	Nov. 15	June 5	6.21	Nov. 1	June 19	3.03	Mar. 13	Apr. 10	2.99
	Nov. 15	Jan. 31	4.48	Jan. 31	Feb. 21	5.28	Apr. 10	May 1	3.44
II	Mar. 29	June 19	3.07	Mar. 29	June 5	5.57	May 8	May 22	3.77
	May 1	May 22	5.15	May 15	June 5	4.84	May 15	June 12	3.97
III	Sept. 20	Oct. 19	3.81	Sept. 20	Oct. 19	5.10	Sept. 20	Nov. 9	1.69
IV	Jan. 2	Feb. 19	6.89	Nov. 27	Jan. 29	3.04	Jan. 2	Feb. 19	2.68
	Apr. 23	June 18	7.89	Jan. 29	Mar. 26	6.73	Feb. 13	June 11	4.67
V	Dec. 29	June 21	5.12	Dec. 29	June 21	2.94	Dec. 29	Mar. 15	3.94
	Jan. 10	Feb. 21	5.76	Jan. 3	Feb. 7	3.90	Mar. 15	Apr. 26	2.21

In general, it was found that in two groups of rabbits examined during the same months, the variations in the mean levels of the three classes of granular leucocytes were similar both as regards the direction of the change and the time at which it occurred. This group resemblance was particularly striking in the case of the marked fluctuations of the eosinophiles but it was also characteristic of the neutrophiles and the basophiles. The results of the two years differed in that the general level of the neutrophile, the basophile, and the eosinophile mean values was higher in the groups examined in 1927–28 than in those observed in 1928–29. Both features of group resemblance and the yearly difference of level were also characteristic of the results pertaining to the red blood cells and the hemoglobin content (1) and the total white, the granular, and the non-granular cells (2). In the case of the total white count and its granular and non-granular components, the period of greatest irregularity of mean values occurred in the late winter and spring months of both years. This was true of the neutrophiles in the second year but to a less extent in the first year. The basophile values were very variable throughout the first year while in the second, the greatest irregularities occurred in the winter months. The fluctuations of the eosinophiles were most pronounced in the late winter and spring months of both years.

In the majority of cases, the physical condition of the rabbits throughout the periods of observation was excellent and gains in body weight were the rule. There were, however, a number of instances of clinical snuffles of slight or moderate degree as well as several cases of ear canker. The effect of these conditions upon the blood count will subsequently be taken up in detail when the results of these and other experiments are considered from the standpoint of the individual animal and of a comparison with snuffles free stock; at present the subject can only be referred to briefly. There were 2 deaths among the 45 rabbits of these experiments, occurring in Group IV; both animals had had snuffles and in one, a chronic nephritis was found at postmortem examination. It appeared that the occurrence of a muco-purulent nasal discharge was most frequently associated with an increase of neutrophiles and in certain instances, of lymphocytes, while in a few cases, higher monocyte values were observed. The persistence of a nasal discharge was not, however, necessarily accompanied by continued high counts. On the other hand, increased counts were observed in rabbits in which no intercurrent infection could be demonstrated by repeated clinical examination and in addition, comparable fluctuations of cell levels were found in both "normal" rabbits and in those with slight or moderate snuffles. The effect of ear canker upon the blood count was not clearly defined. In certain animals, its occurrence coincided with a transitory increase of the neutrophile and lymphocyte cells, while in others, no characteristic alteration of cell levels was observed. Although these conditions of intercurrent infection undoubtedly had an effect upon the blood count, nevertheless, they do not appear to be a factor of major importance with respect to the character of the general variations in cell levels observed over long periods of time from the standpoint of the consecutive mean values of these animal groups.

CONCLUSIONS

Consecutive weekly observations on the neutrophile, the basophile, and the eosinophile counts of the peripheral blood were made on 5 groups of normal rabbits, a total of 45 animals, during a period of 20 months from October, 1927 to July, 1929. Individual groups were examined 8 to 35 weeks.

In the case of the 4 groups followed 13 to 35 weeks, the general trend of the neutrophile cells was towards increased mean values; with the group followed 8 weeks, decreasing values were found. An increase in the mean values of the basophile cells was observed in the two groups of rabbits followed in 1927–28; in the groups of 1928–29, the mean values decreased. The mean values of the eosinophile cells showed no definite trends but the findings were characterized by abrupt and marked fluctuations.

The periods of greatest irregularity in mean neutrophile and eosinophile values occurred in the fall and the late winter and spring months of both years, but in the case of the basophiles, the irregularities were distributed throughout the first year and occurred chiefly in the winter months of the second year.

The major trends and many of the minor fluctuations as well which were observed in the mean cell values of one group of rabbits were also generally seen in another group examined during the same months.

The general levels of the neutrophile, the basophile, and the eosinophile mean values in the groups examined during 1927–28 were higher than in the groups of 1928–29.

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STUDIES IN THE BLOOD CYTOLOGY OF THE RABBIT

V. CONSECUTIVE LYMPHOCYTE AND MONOCYTE OBSERVATIONS ON GROUPS OF NORMAL RABBITS

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Previous papers of this series have contained the results of repeated blood examinations on 5 groups of normal male rabbits with respect to the erythrocyte and hemoglobin determinations (1), the total white cell contents (2), and the neutrophile (pseudo-eosinophile), the basophile, and the eosinophile cell counts (3). The purpose of these experiments was to obtain information on the general character or trend of the spontaneous variations described by the cellular constituents of the peripheral blood over prolonged periods of time, and the data obtained have, in each instance, been presented on the basis of the consecutive weekly mean values of each group. In the present paper, the results on the non-granular cells—the lymphocytes and the monocytes—are reported.

Materials and Methods

A description of the materials and methods employed in these experiments and of the method of analyzing the results obtained has been given in the paper dealing with the observations on the erythrocytes and hemoglobin (1). Suffice it to say here that the period of time covered by the experiments extended from October, 1927 to July, 1929, and in the majority of cases, the blood was examined at weekly intervals. The numbers of examinations were: Group I, 35; Group II, 13; Group III, 8; Group IV, 29; Group V, 26. Each of the first 4 groups comprised 10 and the last, 5 normal male rabbits. The supravital neutral red technic was used in making the differential white cell counts and 100 cells were counted in each specimen.

RESULTS

The consecutive lymphocyte and monocyte determinations on 5 groups of normal rabbits are given in Tables I to V in the form of mean

TABLE I

Group I—10 Rabbits. Consecutive Values for Lymphocytes and Monocytes

Date	Lymphocytes			Monocytes		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
1927-28	*per cmm.*	*per cmm.*	*per cent*	*per cmm.*	*per cmm.*	*per cent*
Oct. 24*..............	2253 ± 185	867	25.97	987 ± 83	388	39.31
Nov. 1...............	1861 ± 92	431	23.16	1092 ± 139	650	59.52
Nov. 8**.............	1929 ± 165	772	40.02	1153 ± 104	487	42.24
Nov. 15..............	2560 ± 105	491	19.18	1085 ± 83	389	35.85
Nov. 22..............	2005 ± 169	794	39.60	1409 ± 185	869	61.67
Nov. 29..............	*2984 ± 210*	*983*	*32.94*	*1209 ± 174*	*817*	*67.63*
Dec. 6..........	*2781 ± 238*	*1117*	*40.17*	*1073 ± 114*	*533*	*49.67*
Dec. 13..............	3171 ± 399	1871	59.00	1178 ± 127	596	50.59
Dec. 20..............	2209 ± 166	778	35.22	1231 ± 127	595	48.33
Dec. 27..............	2554 ± 197	922	36.10	864 ± 77	362	41.90
Jan. 3	2945 ± 333	1559	52.94	1045 ± 52	245	23.44
Jan. 10	3425 ± 256	1202	35.09	967 ± 73	341	35.26
Jan. 17	3429 ± 352	1648	49.11	1025 ± 102	480	46.83
Jan. 24	3511 ± 285	1336	38.05	967 ± 58	270	27.92
Jan. 31	3444 ± 284	1332	38.68	1023 ± 81	380	37.15
Feb. 7...............	3501 ± 263	1235	35.28	1100 ± 106	497	45.18
Feb. 14.......... ...	3070 ± 225	1056	34.40	1201 ± 134	627	52.21
Feb. 21	4671 ± 496	2324	49.75	1218 ± 148	693	56.90
Feb. 28	2507 ± 151	708	28.24	1145 ± 101	474	41.40
Mar. 6...............	2934 ± 183	859	29.28	1117 ± 76	354	31.69
Mar. 13..............	2532 ± 221	1037	40.96	1183 ± 138	648	54.78
Mar. 20..............	2563 ± 166	779	30.39	1084 ± 83	389	35.89
Mar. 27..............	3044 ± 217	1015	33.34	1112 ± 104	488	43.88
Apr. 3...............	3322 ± 335	1571	47.29	1181 ± 127	595	50.38
Apr. 10	3966 ± 337	1578	39.79	1027 ± 123	576	56.09
Apr. 17	3734 ± 245	1149	30.77	1107 ± 121	569	51.40
Apr. 24..............	3073 ± 288	1352	44.00	933 ± 134	627	67.20
May 1...............	4165 ± 316	1483	35.61	933 ± 93	438	46.95
May 8...............	3358 ± 251	1178	35.08	1092 ± 87	407	37.27
May 15..............	3775 ± 190	891	23.60	1071 ± 100	468	43.70
May 22..	3624 ± 169	792	21.85	1245 ± 110	516	41.45
May 29..............	4182 ± 324	1521	36.37	1410 ± 294	1377	97.66
June 5..............	4602 ± 205	959	20.84	1543 ± 169	790	51.20
June 12..............	4455 ± 473	2219	49.81	1270 ± 117	546	42.99
June 19..............	3376 ± 292	1371	40.61	1106 ± 114	536	48.46
Mean.............	3186 ± 83	731	22.94	1125 ± 16	139	12.36
Minimum..........	1861			864		
Maximum..........	4671			1543		

* October 24 and 26.

** November 4 and 9.

TABLE II

Group II—10 Rabbits. Consecutive Values for Lymphocytes and Monocytes

Date	Lymphocytes			Monocytes		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
1928	*per cmm.*	*per cmm.*	*per cent*	*per cmm.*	*per cmm.*	*per cent*
Mar. 29	2210 ± 173	809	36.61	573 ± 35	165	28.80
Apr. 3	3313 ± 262	1227	37.04	677 ± 101	474	70.01
Apr. 10	3073 ± 287	1347	43.83	844 ± 89	416	49.29
Apr. 17	3429 ± 286	1342	39.13	1076 ± 154	724	67.29
Apr. 24	3067 ± 203	950	30.97	853 ± 102	476	55.80
May 1	3257 ± 231	1081	33.19	680 ± 64	299	43.97
May 8	3215 ± 216	1012	31.48	777 ± 83	388	49.94
May 15	2605 ± 247	1158	44.45	721 ± 126	590	81.83
May 22	3960 ± 284	1331	33.61	1028 ± 101	475	46.21
May 29	3758 ± 365	1713	45.58	843 ± 97	455	53.97
June 5	3864 ± 252	1180	30.54	796 ± 70	329	41.33
June 12	3236 ± 244	1144	35.35	1063 ± 110	517	48.64
June 19	3526 ± 222	1040	29.50	1166 ± 66	310	26.59
Mean	3270 ± 87	465	14.22	854 ± 32	173	20.26
Minimum	2210			573		
Maximum	3960			1166		

TABLE III

Group III—10 Rabbits. Consecutive Values for Lymphocytes and Monocytes

Date	Lymphocytes			Monocytes		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
1928	*per cmm.*	*per cmm.*	*per cent*	*per cmm.*	*per cmm.*	*per cent*
Sept. 20	1971 ± 268	1254	63.62	712 ± 113	529	74.30
Sept. 28	2454 ± 287	1345	54.81	780 ± 88	414	53.08
Oct. 10	2178 ± 82	385	17.68	739 ± 80	373	50.47
Oct. 19	2497 ± 142	667	26.71	813 ± 103	482	59.29
Nov. 2	2009 ± 167	784	39.02	797 ± 101	472	59.22
Nov. 9	2352 ± 151	708	30.10	820 ± 64	300	36.59
Nov. 16	1883 ± 110	516	27.40	733 ± 89	415	53.69
Nov. 22	2348 ± 181	850	36.20	895 ± 112	525	58.66
Mean	2212 ± 53	220	9.95	786 ± 13	55	7.00
Minimum	1883			712		
Maximum	2497			895		

values, together with the probable errors of the means, the standard deviations, and the coefficients of variation. The curves in Text-figs. 1 and 2, illustrating the mean values, have been drawn in the form of the percentage deviations of the smoothed means from standard

TABLE IV

Group IV—10 Rabbits. Consecutive Values for Lymphocytes and Monocytes

Date	Lymphocytes			Monocytes		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
1928–29	*per cmm.*	*per cmm.*	*per cent*	*per cmm.*	*per cmm.*	*per cent*
Nov. 27	2616 ± 173	812	31.02	546 ± 39	183	33.56
Dec. 4	2713 ± 153	717	26.44	499 ± 45	212	42.47
Dec. 11	2319 ± 182	854	36.84	452 ± 55	256	56.63
Dec. 18	2168 ± 211	991	45.72	790 ± 171	801	101.35
Dec. 26	2160 ± 186	871	40.31	362 ± 49	229	63.24
Jan. 2	1563 ± 82	383	24.48	456 ± 60	280	61.40
Jan. 8	2040 ± 172	807	39.55	490 ± 90	422	86.08
Jan. 15	1955 ± 84	394	20.13	563 ± 48	224	39.80
Jan. 22	1839 ± 160	751	40.81	704 ± 87	406	57.72
Jan. 29	2470 ± 154	720	29.15	591 ± 73	342	57.84
Feb. 5	1873 ± 144	676	36.09	546 ± 70	330	60.34
Feb. 13	2036 ± 108	505	24.80	826 ± 148	693	83.89
Feb. 19	1907 ± 146	686	35.97	893 ± 101	474	53.08
Feb. 26	2038 ± 139	651	31.96	1066 ± 100	468	43.87
Mar. 12	2007 ± 142	668	33.29	1006 ± 92	431	42.81
Mar. 19	2189 ± 129	604	27.58	857 ± 74	346	40.40
Mar. 26	2290 ± 199	934	40.76	635 ± 66	310	48.85
Apr. 2	1561 ± 185	869	55.70	856 ± 94	443	51.74
Apr. 9	2530 ± 168	787	31.09	1066 ± 98	460	43.15
Apr. 16	2765 ± 233	1092	39.50	709 ± 57	269	37.95
Apr. 23	2971 ± 200	939	31.62	916 ± 99	462	50.44
Apr. 30	2301 ± 183	857	37.23	619 ± 88	413	66.63
May 7	2574 ± 146	686	26.64	699 ± 46	216	30.95
May 14	2514 ± 150	668	26.55	622 ± 62	276	44.34
May 21	1672 ± 93	413	24.71	565 ± 36	159	28.19
May 28	2523 ± 169	752	29.78	479 ± 58	259	54.11
June 4	1960 ± 172	763	38.94	678 ± 62	276	40.77
June 11	1920 ± 153	682	35.53	509 ± 44	196	38.50
June 18	2609 ± 205	860	32.94	530 ± 42	176	33.18
Mean	2210 ± 45	363	16.40	673 ± 24	190	28.20
Minimum	1561			362		
Maximum	2971			1066		

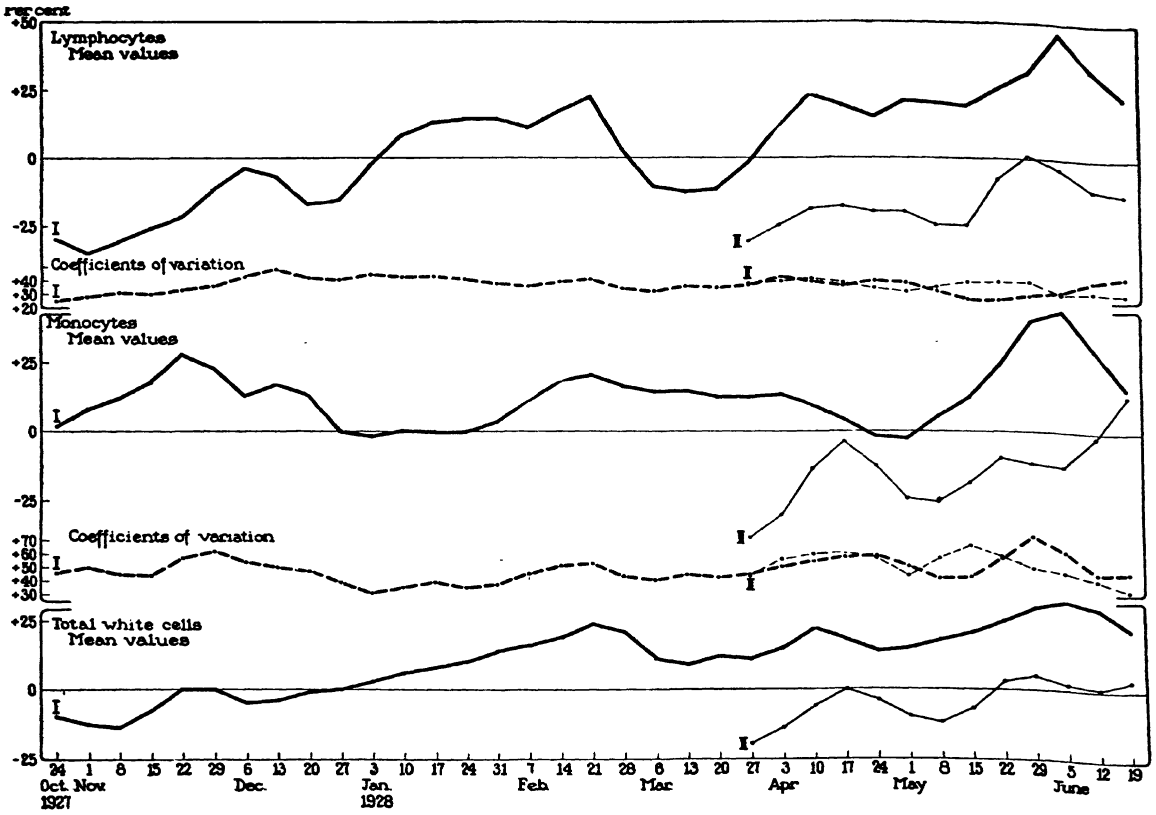
values (1,4); similar curves for the total white cells (2) have been included to facilitate convenient comparisons with their fluctuations. The other curves in the text-figures represent the smoothed coefficients

TABLE V

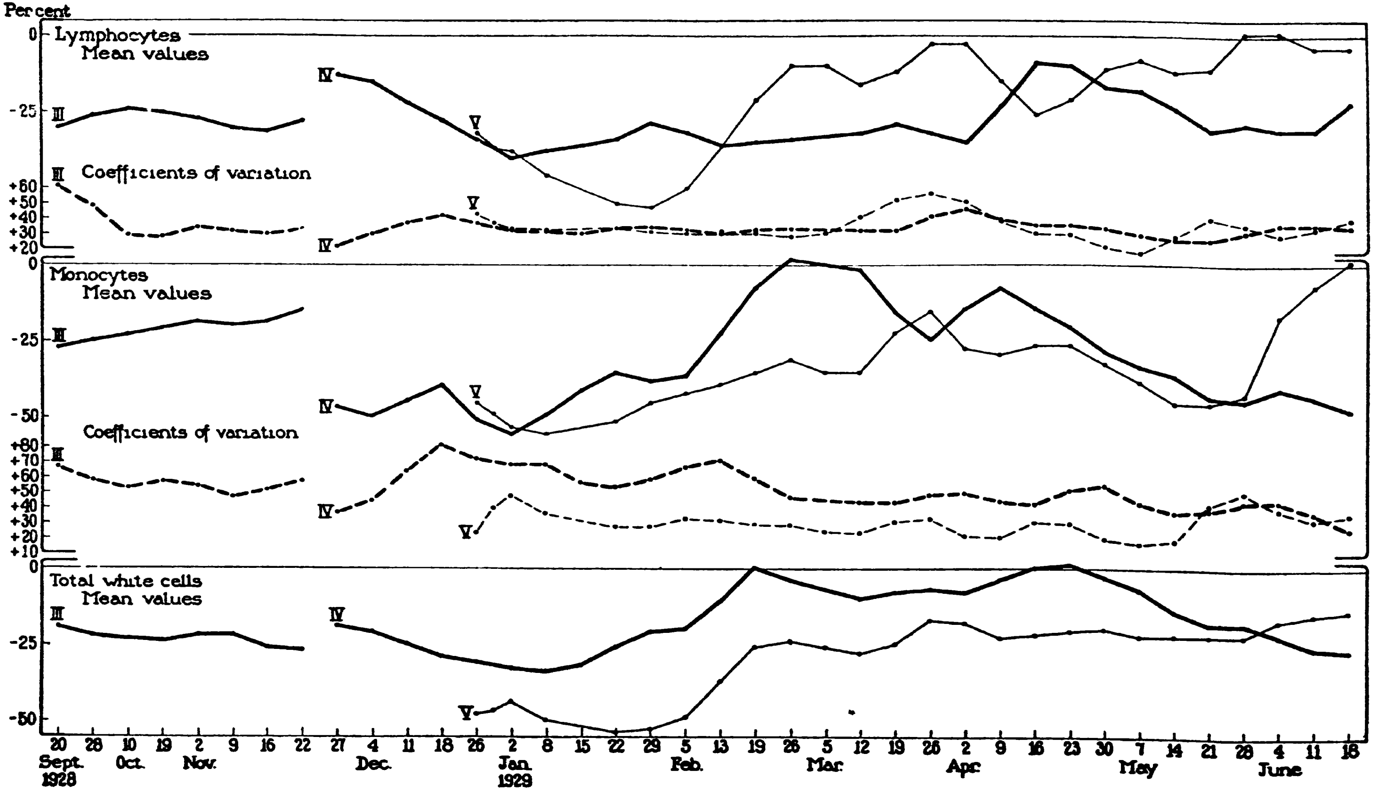
Group V—5 Rabbits. Consecutive Values for Lymphocytes and Monocytes

Date	Lymphocytes			Monocytes		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
1928–29	*per cmm.*	*per cmm.*	*per cent*	*per cmm.*	*per cmm.*	*per cent*
Dec. 29	2310 ± 95	314	13.59	631 ± 92	305	48.34
Dec. 31	1650 ± 206	683	41.39	350 ± 34	113	32.29
Jan. 3	2127 ± 386	1279	60.13	588 ± 65	215	36.56
Jan. 10	1676 ± 130	431	25.72	309 ± 26	86	27.83
Jan. 24	1135 ± 107	353	31.10	544 ± 60	200	36.76
Jan. 31	1503 ± 90	299	19.89	505 ± 46	153	30.30
Feb. 7	1198 ± 139	460	38.40	591 ± 44	146	24.70
Feb. 15	2147 ± 211	698	32.51	605 ± 69	227	37.52
Feb. 21	2304 ± 146	485	21.05	618 ± 47	156	25.24
Mar. 1	2941 ± 328	1087	36.96	723 ± 71	234	32.37
Mar. 8	2820 ± 156	517	18.33	639 ± 47	146	22.85
Mar. 15	2387 ± 167	552	23.13	561 ± 67	223	39.75
Mar. 22	2610 ± 214	710	27.20	816 ± 148	492	60.29
Mar. 29	3092 ± 396	1314	42.50	905 ± 133	441	48.75
Apr. 5	3038 ± 133	440	14.48	671 ± 132	436	64.98
Apr. 12	2668 ± 114	377	14.13	635 ± 54	179	28.19
Apr. 19	1990 ± 226	749	37.64	875 ± 73	242	27.66
Apr. 26	2357 ± 208	688	29.19	523 ± 60	199	38.05
May 3	2927 ± 160	529	18.07	788 ± 31	101	12.82
May 10	2700 ± 117	388	14.37	563 ± 31	101	17.94
May 17	2886 ± 139	459	15.90	524 ± 32	106	20.23
May 24	2219 ± 184	610	27.49	547 ± 85	283	51.74
May 31	3580 ± 605	2006	56.03	531 ± 56	184	34.65
June 7	2913 ± 176	584	20.05	880 ± 60	198	22.50
June 14	2913 ± 173	572	19.64	1023 ± 107	353	34.51
June 21	2944 ± 260	861	29.25	1007 ± 125	414	41.11
Mean	2424 ± 80	607	25.04	652 ± 23	176	26.99
Minimum	1135			350		
Maximum	3580			1023		

of variation of the means. The standard values used in this comparison of mean values are: lymphocytes, 3050 per cubic millimeter; monocytes, 1000 per cubic millimeter; total white blood cells, 9560 per cubic millimeter.



TEXT-FIG. 1. Mean values for consecutive lymphocyte and monocyte determinations as percentage deviations from standard values. 1927–28.



TEXT-FIG. 2. Mean values for consecutive lymphocyte and monocyte determinations as percentage deviations from standard values. 1928–29.

DISCUSSION AND SUMMARY

The results of these experiments will be discussed in chronological order. It will be noted that the observations on Group II were made during the spring of 1928 at which time Group I was still being examined, and that Groups IV and V were followed during the winter and spring months of 1928 and 1929.

Group I was examined at weekly intervals from October 24, 1927 to June 24, 1928 (Table I). From the curves in Text-fig. 1, which illustrate the lymphocyte and monocyte mean values in the form of percentage deviations from standard values as given above, it will be seen that the mean numbers of lymphocytes were considerably increased above their low initial level by the end of the experiment. This change was accomplished gradually and in a fairly consistent fashion with the exception of a minor drop of the values in December and June and an abrupt and pronounced fall at the end of February and the first half of March. In the case of the monocytes, the general level of which was continuously above the standard value, no such sustained increase of mean values was observed. The curve representing these cells contains 3 major upward swings which occurred in November, in February and March, and in May. During certain periods, as in October and November, and again in May and June, the lymphocyte and monocyte curves follow the same general trend. At other times, however, this parallel relationship did not hold. In January, the lymphocyte curve describes a sharp rise while the monocyte curve is stationary and in February, March, and April, when an abrupt fall of the lymphocyte curve is followed by a pronounced rise, the monocyte curve describes a slight but protracted decline. It will be seen by the curve in Text-fig. 1, that the total white cell count was considerably increased during the experiment, and a comparison with the lymphocyte curve shows that its fluctuations are frequently reflected in those of the total white cells. Although the coefficients of variation of the lymphocyte means (Table I) are of a high order of magnitude, the curve of the smoothed values (Text-fig. 1) is quite regular. The monocyte coefficients (Table I) are slightly higher than those of the lymphocytes and their smoothed curve (Text-fig. 1) is more irregular.

Group II was examined from March 29 to June 19, 1928 (Table II). The curves representing its lymphocyte and monocyte means (Text-fig. 1) bring out clearly the lower numerical levels of both classes of cells as compared with those of Group I. In the case of the lymphocytes the general forms of the curves of Groups I and II are very similar but with the monocytes, the resemblance is less striking.

The lymphocyte and total white cell curves are, on the whole, much alike. There are certain portions of the latter curve, however, which resemble the monocyte more than the lymphocyte curve, as for example, that covered by the last two observations. The coefficients of variations of the lymphocyte and monocyte means of Group II (Table II) are of the same order of magnitude as those of Group I and their smoothed curves (Text-fig. 1) present the same general appearance.

Group III was examined from September 20 to November 22, 1928 (Table III). From the curves in Text-fig. 2, it will be seen that the levels of both the lymphocyte and the monocyte mean values were of the same order as those of Group II rather than those of Group I. Only minor changes in the values of Group III were observed. The monocyte curve describes a gradual comparatively slight rise while the lymphocyte curve shows no clearly defined trend. The total white cell curve describes a slight downward trend and it is almost a mirror image of the monocyte curve. With the exception of the first two points of the lymphocyte curve, the curves of the smoothed coefficients of variation of the lymphocyte and monocyte means (Text-fig. 2) are comparable to those of Groups I and II.

Group IV was examined from November 27, 1928 to June 19, 1929 (Table IV). The general level of the lymphocyte mean values was comparable to the results in Groups II and III and to the first quarter of the findings of Group I. During December, the trend of mean values was decreasing as shown by the falling curve (Text-fig. 2); during January, February, and March, a low level was fairly constantly maintained; in April, an abrupt increase occurred and this was succeeded by a second decrease. The curve representing the monocytes (Text-fig. 2) shows first that the general level of mean values was usually lower than that of Group I and was more comparable to those of Groups II and III. From very low levels in December and early January, the pronounced upward swing of the curve in February, March, and April indicates the increase of monocytes during this time, and its subsequent descent illustrates the eventual drop in the numbers of these cells. The general trend of the lymphocyte and the monocyte means is similar in the beginning and during the last third of the experiment, but during much of the time, the increase of monocytes was accompanied by practically no change in the level of lymphocyte mean values. In general form, the total white cell curve (Text-fig. 2) has many points of resemblance with the monocyte as well as with the lymphocyte curve.

The coefficients of variation of the lymphocyte means (Table IV, Text-fig. 2) which are on the whole quite uniform, are of a similar order of magnitude to those of the other groups. The coefficients of the monocytes (Table IV, Text-fig. 2) are somewhat more variable and tend to be slightly higher than those of the other groups.

Group V was examined from December 29, 1928 to June 21, 1929 (Table V). The mean lymphocyte values as shown by the curve in Text-fig. 2 were on the whole higher than those of Group IV examined during the same months, but they did not equal the high levels of Group I examined the previous year. In its first portion, the curve representing these findings resembles that of Group IV, but its rise was initiated in February and from then onward to the end of the experiment, a comparatively high level prevailed. The rise of the Group IV curve, which was of short duration, occurred in March and April. In respect to the maintenance of higher values in the spring months, Group V resembled Groups I and II. The monocyte curves of Groups IV and V are, on the whole, similar in general form until the end of the experiment (Text-fig. 2); during June, the curve for Group V describes an abrupt and marked rise while that of Group IV continues its downward trend. The general level of the Group V mean values was slightly lower than those of Group IV. It will be noted by comparing their respective curves, that the movements of the lymphocyte and monocyte mean values are generally similar in direction during the first two-thirds of the experiment. In April and May, however, the lymphocyte curve is rising while the monocyte curve is falling; in June the lymphocyte curve is stationary at a high level while the monocyte curve shows an abrupt rise. On the whole, the general contour of the total white cell curve of Group V (Text-fig. 2) resembles the lymphocyte more than the monocyte curve. During April and May, however, when its level continues to be quite regularly maintained, the lymphocytes are rising and the monocytes are falling; in June, when it is undergoing a gradual and comparatively slight rise, the lymphocytes are high and the monocytes are very high.

The coefficients of variation of the lymphocyte means of Group V (Table V, Text-fig. 2) are entirely comparable to those of Group IV; those of the monocyte means (Table V, Text-fig. 2) on the other hand, are considerably smaller and are generally more uniform. As far as general level is concerned, the monocyte coefficients of Group V are the lowest of any of the 5 groups.

There are certain features of these results which merit special attention. In the first place, the general levels of both lymphocyte and monocyte mean values during 1927–28 were on the whole higher than in 1928–29, as was found with the red cells and hemoglobin (1), the total white cells (2), and the neutrophiles, the basophiles, and the eosinophiles (3). Secondly, the parallelism shown by two groups examined during the same months with respect to the direction of a change in the level of mean cell values and the time of its occurrence, which was characteristic of the other cells and of the hemoglobin content, was less pronounced in the case of the lymphocytes and monocytes. With Groups I and II, the lymphocyte curves are very similar but those of the monocytes are less alike; with Groups IV and V, the monocyte curves up to their last periods have a general resemblance to each other while the lymphocyte curves are frequently dissimilar. Thirdly, the general trend of cell level shown by the lymphocytes was one of increasing values in the late winter and spring months while lower values prevailed in the fall and winter. In the case of the monocytes, a similar but less consistent trend toward higher numerical values was observed and in addition to a late winter and spring rise, there was some indication that higher counts were characteristic of the early winter months (Group I, November; Group III, rise of curve from a lower September to a higher November level; Group IV, fall of curve from a higher November to a lower December level). Fourthly, the period of greatest irregularity in the lymphocyte mean values occurred in the late winter and spring months of both years; with the monocytes, on the other hand, this feature was observed in the fall and early summer months of the first year and during the late winter, the spring and the early summer months of the second year.

The significance to be attached to the major changes in the levels of lymphocyte and monocyte mean values is indicated by the following examples of the ratios of the differences of various means to their probable errors.

Group number	Lymphocytes			Monocytes		
	Dates of mean values		Ratio	Dates of mean values		Ratio
I	Oct. 24	June 19	3.26	Oct. 24	June 5	2.94
	Feb. 21	May 20	4.03	May 1	June 5	3.16
	Mar. 20	June 5	7.72			
II	Mar. 29	May 22	5.26	Mar. 29	June 19	7.91
III	Sept. 20	Oct. 19	1.74	Sept. 20	Nov. 22	0.92
IV	Nov. 27	Jan. 2	6.61	Dec. 26	Feb. 26	6.34
	Jan. 2	Apr. 23	3.45	Feb. 26	June 18	4.96
	Apr. 23	June 11	2.46	Feb. 26	Mar. 26	3.59
V	Dec. 29	June 14	3.06	Dec. 26	June 21	2.43
	Jan. 24	Mar. 8	8.92	May 17	June 14	4.46
	Apr. 19	June 7	3.23			

The question of the numerical relationship of lymphocytes and monocytes in the peripheral blood has received much attention. In so far as the results on these groups of normal rabbits are concerned, the present findings indicate that a constant and consistent relationship between these cells does not obtain over long periods of time. This feature of the observations may be best appreciated by comparing the respective curves representing the consecutive weekly mean values of these cells (Text-figs. 1 and 2). In certain portions, both curves show similar changes in level during the same weeks, indicating that both classes of cells have undergone analogous numerical changes (Group I, November, May, June; Group IV, January, April, May; Group V, February, April). In other portions, however, the curves move in opposite directions (Group I, December, January, April; Group II, June; Group III, October; Group IV, December, February; Group V, May, June). It is evident, however, that before final conclusions on the lymphocyte-monocyte numerical relationship can be drawn, the results of experiments of this type must be analyzed from the standpoint of the individual animal.

CONCLUSIONS

Consecutive weekly observations on the lymphocyte and monocyte counts of the peripheral blood were made on 5 groups of normal rabbits, a total of 45 animals, during a period of 20 months from October, 1927 to July, 1929. Individual groups were examined 8 to 35 weeks. The results are analyzed on the basis of the mean group values of each week.

In the case of the 4 groups followed 13 to 35 weeks, there was a general tendency for the lymphocyte mean values to become increased; with the group observed 8 weeks, the level of mean values showed little change. The general trend of the monocyte mean values was also in the direction of higher levels but it was less pronounced than that of the lymphocytes.

The period of greatest irregularity in the mean values of the lymphocytes was in the late winter and spring months of both years. With the monocytes, periods of fluctuating values occurred in the fall of 1927, the spring and early summer of 1928, and in the late winter, spring, and early summer of 1929.

There was a certain degree of parallelism in the case of two groups examined during the same months with respect to the direction and time of occurrence of a change in the level of lymphocyte and monocyte mean values.

The general levels of lymphocyte and monocyte mean values in the groups examined during 1927–28 were higher than in the groups of 1928–29.

The results based upon the trends of mean group values obtained from consecutive weekly observations showed no evidence of a consistent numerical relationship between lymphocytes and monocytes.

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THE INFLUENCE OF ULTRA-VIOLET RADIATION ON THE WEIGHT OF ADULT RABBITS, NORMAL AND SYPHILITIC

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During the course of an experiment previously reported (1), it was noted that rabbits exposed to ultra-violet radiation under certain conditions did not appear to be in as good physical condition as animals living in an environment from which all light was excluded and were more susceptible to intercurrent infections. With these facts in mind, the experiment was repeated with a view to determining the influence of ultra-violet irradiation on body weight, the chemical composition of the blood, and susceptibility to intercurrent infections of normal and syphilitic animals and on the reaction to syphilitic infection. The present paper will be limited to a report of the results obtained for body weight and susceptibility to intercurrent infections.

Material and Methods

In the experiment to be reported, 60 male rabbits between 6 and 8 months of age were used. The animals were divided into 6 comparable groups of 10 each, and from the beginning of the experiment on November 27, 1928, until June 15, 1929, these animals were kept in a room from which all light was excluded. They were caged separately, and fed the routine diet of hay, oats, and cabbage. The temperature of the dark room was satisfactorily maintained at 70° to 75°F. and the humidity varied with that of the outside air. The animals were weighed at weekly intervals.

Beginning December 5, 1928, 30 animals, listed as Groups II, IV, and VI were exposed for an hour each day to the unfiltered radiations of a quartz-mercury arc lamp (80 volts, 4.2 amperes) at a distance of 4.5 meters. The doors of the cages were open to permit free access of light but no depilatory measures were used and at no time throughout the experiment could there be detected evidence of dermatitis, conjunctivitis, or any other inflammatory process which might be attributed to the action of ultra-violet radiation. The other animals, Groups I, III, and V, remained in the dark room throughout the experiment. Group I served as a control for Groups III and V, and Group II, as the control for Groups IV and VI.

A summary of experimental procedures, according to groups, is given in the following table:

Group	Number of animals	Dark room	Ultra-violet irradiation started	Inoculated with *T. pallidum*	Termination of experiment
I	10	Nov. 27, 1928			June 15, 1929
II	10	Nov. 27, 1928	Dec. 5, 1928		June 15, 1929
III	10	Nov. 27, 1928		Dec. 18, 1928	June 15, 1929
IV	10	Nov. 27, 1928	Dec. 5, 1928	Dec. 18, 1928	June 15, 1929
V	10	Nov. 27, 1928		Jan. 24, 1929	June 15, 1929
VI	10	Nov. 27, 1928	Dec. 5, 1928	Jan. 24, 1929	June 15, 1929

All animals received some light from two other sources. For a brief period each day a 30 Watt amber tinted Mazda lamp was used in the dark room for cleaning cages, feeding, and making certain necessary observations. The animals were also exposed to diffuse filtered sunlight for a brief period once every 2 weeks when they were brought into the laboratory for bleeding purposes. During the remaining time all animals were kept in the dark room.

The mean values contained in Tables I to VI inclusive have been smoothed by the formula $\frac{a + 2b + c}{4}$ and are presented in the graphs (Text-figs. 1 to 3) in terms of per cent variation from a standard mean value, using for this purpose 2200 gm.

RESULTS

The results of the observations made in this experiment are presented in the form of tabulated summaries, Tables I to VIII, which are supplemented by a series of graphs, Text-figs. 1 to 3.

TABLE I

Gross Body Weight. Normal Dark Room

Date	Group mean	Smoothed net variation	Per cent variation
1928-29	*gm.*	*gm.*	
Nov. 30	1940	−260	−11.8
Dec. 6	2043	−167	− 7.6
Dec. 12	2088	−113	− 5.1
Dec. 20	2132	− 82	− 3.7
Dec. 28	2121	− 61	− 2.8
Jan. 5	2182	− 21	− 0.9
Jan. 12	2232	+ 17	+ 0.8
Jan. 18	2222	+ 32	+ 1.5
Jan. 25	2253	+ 59	+ 2.7
Feb. 5	2309	+105	+ 4.8
Feb 9	2350	+154	+ 7.0
Feb. 16	2409	+199	+ 9.0
Feb. 22	2431	+186	+ 8.8
Mar. 6	2273	+117	+ 5.3
Mar. 16	2290	+ 94	+ 4.3
Mar. 23	2325	+114	+ 5.2
Mar. 29	2315	+ 88	+ 4.4
Apr. 6	2197	+ 33	+ 1.5
Apr. 14	2225	+ 25	+ 1.1
Apr. 19	2262	+ 61	+ 2.8
Apr. 27	2295	+ 98	+ 4.5
May 11	2342	+138	+ 6.3
May 17	2355	+103	+ 4.7
May 25	2162	− 1	0
June 1	2120	− 58	− 2.6
June 8	2167	− 37	− 1.7
June 15	2200	0	0
Group mean.......	2231	+ 31	+ 1.4

Dec. 6 to Jan. 25—14.5.

Mean gain—13.2.

Final net gain—11.8

TABLE II

Gross Body Weight. Normal Ultra-Violet

Date	Group mean	Smoothed net variation	Per cent variation
1928-29	*gm.*	*gm.*	
Nov. 30	1865	−335	−15.7
Dec. 6	1970	−194	− 8.8
Dec. 12	1998	−150	− 7.0
Dec. 20	2084	−133	− 6.0
Dec. 28	2103	− 77	− 3.5
Jan. 5	2216	− 10	− 0.4
Jan. 12	2228	+ 34	+ 1.5
Jan. 18	2265	+ 46	+ 2.1
Jan. 25	2236	+ 59	+ 2.6
Feb. 5	2322	+ 75	+ 3.4
Feb. 9	....	.....	......
Feb. 16	2220	+104	+ 4.7
Feb. 22	....	.....	......
Mar. 6	2453	+186	+ 8.8
Mar. 16	2420	+106	+ 4.8
Mar. 23	....	.....	......
Mar. 29	....	.....	......
Apr. 6	....	.....	......
Apr. 14	2031	− 63	− 2.9
Apr. 19	....	.....	......
Apr. 27	2065	−133	− 6.0
May 11	2109	− 81	− 3.6
May 17	2193	− 31	− 1.3
May 25	2180	− 47	− 2.1
June 1	2059	− 98	− 4.5
June 8	2112	−114	− 5.2
June 15	2063	−137	− 6.2
Group mean.......	2152	− 48	− 2.2

Dec. 6 to Jan. 25—18.3.
Mean gain—13.5.
Final net gain—9.5.

TABLE III

Gross Body Weight. Dark Room. T. pallidum Inoculated on December 12, 1928

Date	Group mean	Smoothed net variation	Per cent variation
1928–29	*gm.*	*gm.*	
Nov. 30	1902	−197	− 8.9
Dec. 6	1955	−119	− 5.4
Dec. 12	2180	− 98	− 4.5
Dec. 20	2000	− 63	− 2.9
Dec. 28	2230	+ 31	+ 1.3
Jan. 5	2240	+ 27	+ 1.2
Jan. 12	2342	+168	+ 7.6
Jan. 18	2387	+174	+ 7.9
Jan. 25	2357	+193	+ 8.8
Feb. 5	2397	+211	+ 9.6
Feb. 9	2422	+211	+ 9.6
Feb. 16	2405	+230	+10.5
Feb. 22	2415	+247	+11.2
Mar. 6	2485	+230	+10.5
Mar. 16	2405	+213	+ 9.7
Mar. 23	2425	+214	+ 9.7
Mar. 29	2417	+213	+ 9.7
Apr. 6	2400	+245	+11.2
Apr. 14	2425	+274	+12.4
Apr. 19	2470	+298	+13.5
Apr. 27	2500	+327	+14.9
May 11	2500	+321	+14.8
May 17	2567	+240	+10.9
May 25	2450	+108	+ 4.8
June 1	2295	+ 25	+ 1.1
June 8	2215	+ 6	+ 0.2
June 15	2200	0	0
Group mean.......	2333	+133	+ 6.0

TABLE IV

Gross Body Weight. Ultra-Violet. T. pallidum Inoculated on December 12, 1928

Date	Group mean	Smoothed net variation	Per cent variation
1928–29	*gm.*	*gm.*	
Nov. 30	1852	−248	−11.2
Dec. 6	1900	−183	− 8.7
Dec. 12	2017	−101	− 4.6
Dec. 20	2065	−132	− 6.0
Dec. 28	2127	− 87	− 4.3
Jan. 5	2135	− 56	− 2.4
Jan. 12	2180	− 21	− 0.9
Jan. 18	2212	+ 12	+ 0.5
Jan. 25	2245	+ 40	+ 1.8
Feb. 5	2260	+ 41	+ 1.8
Feb. 9	2212	+ 14	+ 0.6
Feb. 16	....	.....	......
Feb. 22	2175	− 24	− 1.1
Mar. 6	....	.....	......
Mar. 16	2134	− 66	− 3.0
Mar. 23	2093	− 87	− 3.9
Mar. 29	2142	− 67	− 3.0
Apr. 6	2156	− 53	− 2.4
Apr. 14	2134	− 71	− 3.2
Apr. 19	....	.....	......
Apr. 27	....	.....	......
May 11	2090	−108	− 4.9
May 17	....	.....	......
May 25	....	.....	......
June 1	2053	−147	− 6.7
June 8	....	.....	......
June 15	....	.....	......
Group mean.......	2115	− 85	− 3.9

TABLE V

Gross Body Weight. Dark Room. T. pallidum Inoculated on January 24, 1929

Date	Group mean	Smoothed net variation	Per cent variation
1928–29	*gm.*	*gm.*	
Nov. 30	1932	−268	−12.2
Dec. 6	2162	−178	− 8.1
Dec. 12	2222	+ 15	+ 0.7
Dec. 20	2255	+ 56	+ 2.4
Dec. 28	2295	+ 91	+ 4.1
Jan. 5	2325	+130	+ 5.9
Jan. 12	2380	+174	+ 7.9
Jan. 18	2412	+212	+ 9.6
Jan. 25	2447	+245	+11.1
Feb. 5	2477	+274	+12.4
Feb. 9	2497	+303	+13.8
Feb. 16	2542	+305	+13.9
Feb. 22	2442	+254	+11.5
Mar. 6	2387	+208	+ 9.5
Mar. 16	2417	+204	+ 9.4
Mar. 23	2395	+191	+ 8.7
Mar. 29	2357	+130	+ 5.9
Apr. 6	2312	+128	+ 5.8
Apr. 14	2332	+127	+ 5.8
Apr. 19	2332	+146	+ 6.6
Apr. 27	2391	+163	+ 7.5
May 11	2339	+148	+ 6.7
May 17	2322	+103	+ 4.7
May 25	2230	+ 50	+ 2.3
June 1	2218	+ 44	+ 2.0
June 8	2290	+ 90	+ 4.1
June 15	....	......	......
Group mean.......	2335	+135	+ 6.1

TABLE VI

Gross Body Weight. Ultra-Violet. T. pallidum Inoculated on January 24, 1929

Date	Group mean	Smoothed net variation	Per cent variation
1928–29	*gm.*	*gm.*	
Nov. 30	1922	−258	−11.5
Dec. 6	2061	−168	− 7.6
Dec. 12	2086	− 96	− 4.2
Dec. 20	2183	− 39	− 1.7
Dec. 28	....	.....	......
Jan. 5	2194	− 2	− 0.1
Jan. 12	2222	+ 22	+ 1.0
Jan. 18	2250	+ 48	+ 2.2
Jan. 25	2269	+ 75	+ 3.4
Feb. 5	2303	+ 81	+ 3.7
Feb. 9	2250	+ 68	+ 3.1
Feb. 16	2272	+ 69	+ 3.1
Feb. 22	2266	+ 65	+ 3.0
Mar. 6	2267	+ 69	+ 3.1
Mar. 16	2277	+ 74	+ 3.4
Mar. 23	....	.....	......
Mar. 29	2275	+ 45	+ 2.0
Apr. 6	....	.....	......
Apr. 14	....	.....	......
Apr. 19	2155	+ 40	+ 1.9
Apr. 27	....	.....	......
May 11	....	.....	......
May 17	2025	− 48	− 2.2
May 25	....	.....	......
June 1	2002	−148	− 6.7
June 8	1922	−212	− 9.6
June 15	....	.....	......
Group mean.......	2168	− 32	− 1.5

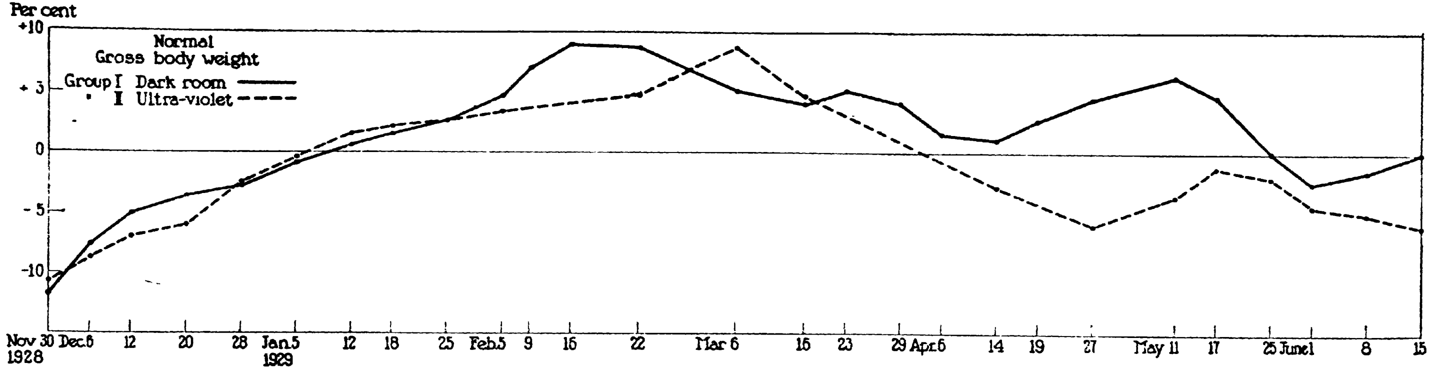
TABLE VII

Per Cent Gain from Initial Weight

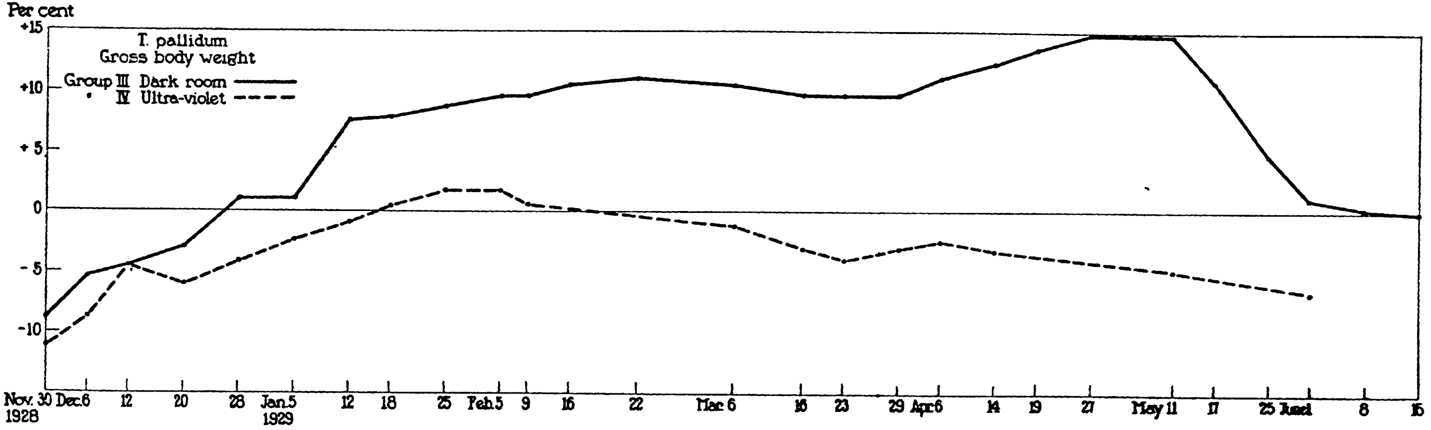
Dark room				Ultra-violet			
Group	Jan. 25, 1929	Mean	Final	Group	Jan. 25, 1929	Mean	Final
I	16.1	15.0	13.4	II	19.9	15.4	10.6
III	24.8	22.6	15.1	IV	21.2	14.2	10.8
V	26.6	20.8	18.5	VI	18.1	12.7	0.0

TABLE VIII

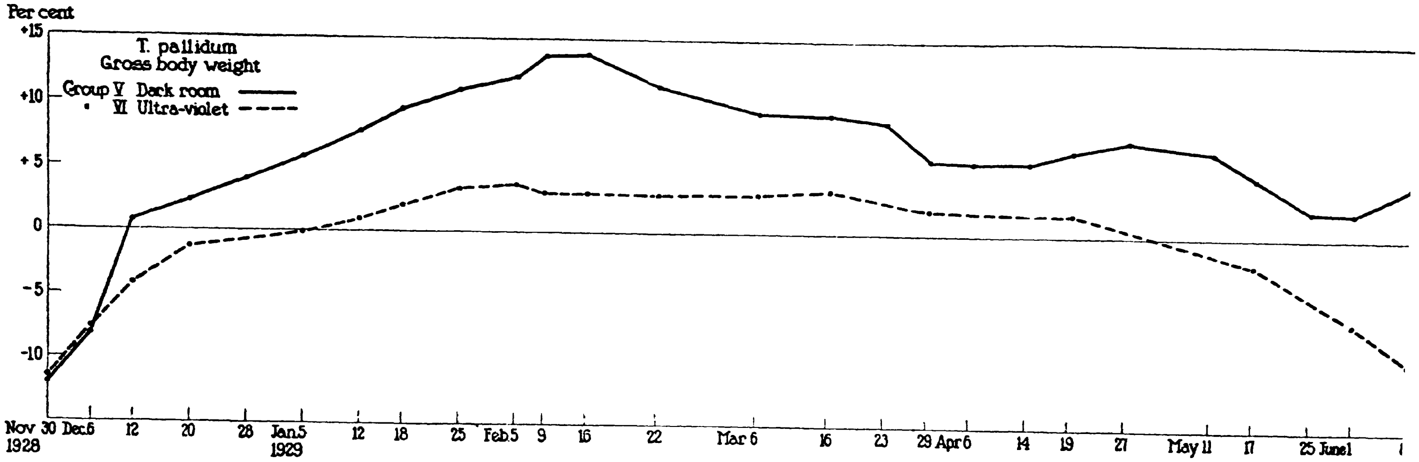
Dark room		Ultra-violet	
Group	Survivals	Group	Survivals
I	10	II	6
III	10	IV	8
V	9	VI	9



TEXT-FIG. 1



TEXT-FIG. 2


Per cent
+15
+10
+5
0
-5
-10
T. pallidum
Gross body weight
Group V Dark room
VI Ultra-violet
Nov 30
1928
Dec.6
12
20
28
Jan.5
1929
12
18
25
Feb.5
9
16
22
Mar 6
16
23
29
Apr 6
14
19
27
May 11
17
25
June1

TEXT-FIG. 3

DISCUSSION AND CONCLUSIONS

In taking up the discussion of the results recorded above, attention should be directed to the fact that in these experiments the only light of any consequence which was received by the irradiated animals was that derived from the quartz mercury lamp. These experiments differ, therefore, from others in which animals receiving unfiltered or diffuse filtered sunlight are given an additional ultra-violet irradiation. A further condition to which attention should be called is the fact that during the course of these experiments, there was a marked deterioration of the lamp used, so that the energy delivered during the latter part of the experiment was much less than at the beginning. It is regretted that the actual change and the rate of decrease can not be given accurately. But throughout the entire period of the experiment, all animals lived in a room from which all extraneous light was excluded, the only evironmental variation being the exposure of Groups II, IV, and VI to ultra-violet radiation for an hour each day at a distance of 4.5 meters.

Considering the results for normal animals first, it will be seen by a comparison of the figures for body weight that from the beginning of the experiment on November 30, 1928, until January 25, 1929, both Groups I and II showed a gradual increase in body weight. Following this, the dark group exhibited a rather sharp increase in weight reaching a maximum on February 16. The increase in the ultra-violet group of animals was more gradual, their maximum weight being attained on March 6. From these high values both groups showed a loss in weight, the group exposed to ultra-violet radiation losing more than animals living in total darkness. After March 16 and throughout the remainder of the experiment, the general level of body weight of the ultra-violet group was below that of the dark group. The latter group exhibited a secondary increase in weight during the first week of May, this same increase being reflected in the ultra-violet animals although at a much lower level. From these levels, both groups decreased in weight, the ultra-violet animals showing a more gradual decline while the dark group showed a slight increase in weight during the last 2 weeks of the experiment.

The difference between the two groups of animals with respect to susceptibility to intercurrent disease (Table VIII) is in agreement with the results obtained for body weight. This is clearly shown by the occurrence of 4 deaths among the irradiated animals and none among those which received no ultra-violet light. Postmortem examination showed that death was due in all cases to pneumonia.

The effects of irradiation on syphilitic rabbits were essentially the same as on normal animals. The animals of Groups III and IV (Tables III and IV; Text-fig. 2) were inoculated with the Nichols strain of *T. pallidum* on December 24, 1928. All other conditions were the same as for Groups I and II. Except for the values obtained on December 12, at which time both groups coincided, the gross body weights for animals exposed to ultra-violet radiation was far below that maintained by animals living in total darkness. On May 6, there was a difference of 20 per cent between the mean values obtained for these two groups of animals. While the course of syphilis in the two groups of animals was moderately severe, yet no deaths were recorded as resulting from the specific infection. However, at the end of the experiment, all animals of Group III were alive while 2 died in Group IV, deaths being due to pneumonia.

The animals in Groups V and VI were inoculated on January 24, 1929, with the same strain of *T. pallidum* as were Groups III and IV. In general, it can be stated that the syphilitic infection in these animals was less severe than that found in Groups III and IV. The mortality rate from non-specific infection was the same in both Groups V and VI.

From the beginning of the experiment until December 6, the animals in Group VI showed a higher gross body weight than those in Group V, but from this time on the animals in Group VI maintained a level below that of Group V. In these two groups of animals, it will be noted that the difference in weight is not as great as that found to exist between Groups III and IV.

If the gain in weight is calculated in per cent of the original or first recorded weights (Table VII), essentially the same results are demonstrable. At the end of the first 7 weeks of the experiment, January 25, 1929, the animals in Group II showed a greater per cent gain in weight than did the control animals in Group I. It will also be noted that the mean per cent gain was slightly higher for animals exposed to ultra-violet radiation than for those animals in the dark room. The final weight for the ultra-violet group was less than for those animals in the dark room. In the 4 other groups the per cent gain was less in animals exposed to ultra-violet light than in those remaining in total darkness; in Group VI the final weight was exactly the same as the original.

Another factor which influences all calculations is the mortality rate among these 6 groups of animals (Table VIII). Seven deaths occurred among animals remaining in the dark room. The deaths in each group were due to pneumonic infections.

SUMMARY AND CONCLUSION

In summarizing the results obtained for these 6 groups of animals, the following conclusions may be drawn:

Normal rabbits living in total darkness and exposed to ultra-violet radiation at regular intervals showed a more rapid rate of increase in weight than animals living under the same condition, but after an initial period of rapid increase, the irradiated animals maintained a lower body weight than those living in the dark.

Under the same conditions, animals inoculated with *T. pallidum* and exposed to ultra-violet light maintained a lower weight than the corresponding control groups living in total darkness.

Furthermore, the mortality rate from pneumonic infection was found to be greater in animals exposed to ultra-violet radiation than in those living entirely in the dark.

It is evident, therefore, that, under the conditions given, ultra-violet radiation was detrimental rather than beneficial.

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THE ORIGIN AND FATE OF TWO TYPES OF MULTINUCLEATED GIANT CELLS IN THE CIRCULATING BLOOD

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Plates 10 and 11

(Received for publication, March 13, 1930)

In the course of studies in the experimental production of giant cells in the tissues, reported by Doan, Sabin and Forkner (1), the writer discovered a procedure by which giant cells could be made to appear in the blood stream. This new material gave valuable evidence concerning the formation of giant cells, more especially of those of the so called foreign body and epithelioid types.

As far as can be ascertained, no one has hitherto reported a method to induce an appearance of giant cells in the blood, and no such cells have been found there, except megakaryocytes, which were reported by Oelhafen (2) and Naegeli (3) in 1914, by Minot (4) in 1922, by Sabin (5) in 1923, and by others. The usual condition in which they have been found is myelogenous leucemia.

Haythorn (6), in a recent review, has classified giant cells as follows: (a) Langhans' giant cells, (b) foreign body giant cells, (c) osteoclasts, (d) megakaryocytes, (e) muscle giant cells, (f) giant cells of nervous tissue, and (g) true tumor giant cells. It is relative to the first two of these types that this paper is concerned.

As early as 1868 Langhans (7), working with fresh tissues, demonstrated large multinucleated cells obtained from tuberculous lesions. For the most part the nuclei were arranged around the periphery of the cell, were usually round or oval, had sharp outlines and generally contained nucleoli. He stated that the protoplasm of such a giant cell was pale, homogeneous, or finely granular, with the center usually clear. This type of cell has since been designated the "Langhans' giant cell." However, some confusion has arisen because he also described another type of giant cell which had nuclei distributed throughout the cytoplasm.

For reasons given by Doan, Sabin and Forkner (1), the term "*epithelioid giant cell*" has been proposed to replace the often misunderstood term of Langhans' giant cell. This concept of the "epithelioid giant cell" is based on new information obtained through the methods of studying living cells with supravital (Simpson, 8)

(Sabin, 5) and vital stains. It was first demonstrated by Sabin, Doan and Cunningham (9) that the epithelioid cell is derived from the monocyte. The supravital technique showed that in the monocyte, surrounding its centrosphere, is a characteristic *rosette of vacuoles, stainable in the living state with neutral red.* An accentuation of this normal structure of the monocyte produces an epithelioid cell. Cunningham, Sabin, Sugiyama, and Kindwall (10) then confirmed the findings of other investigators that the Langhans' giant cell is a multinucleated epithelioid cell.

In general there have been two opinions concerning the types of cells under discussion. One group of investigators, among them Ziegler (11), Kockel (12), Hektoen (13), Maximow (14), and Medlar (15), believe that the Langhans' giant cell is merely a foreign body giant cell occurring in tuberculosis. Krückmann (16) studied giant cells in tuberculosis, about parasites and foreign materials, and in tumors. He concluded that microscopic means were not available by which foreign body giant cells and Langhans' giant cells could be distinguished. Langhans (7), Jacobson (17), Lubarsch (18) and others, however, believe that the giant cell of tuberculous tissue can be distinguished from all other kinds.

Concerning the mode of origin of the foreign body and epithelioid giant cells there has been some disagreement. Weigert (19), Baumgarten (20), Bakács (21), Lubarsch (18), and others were of the opinion that giant cells of tuberculous lesions were formed by continued nuclear division. Krauss (22), Mallory (23) and Wells (24) are among those who favor fusion of individual cells as the explanation for the formation of the types of cells under discussion. Lewis and Webster (25) in 1921 believed that epithelioid giant cells were formed by amitosis of the nucleus, but a later paper by Lewis (26) takes the opposite view, that they are formed by fusion of epithelioid cells.

Materials and Methods

The experiments reported in this communication have been carried out with the use of the methods of supravital and vital staining. Because of some recent minor modifications in the methods used in Dr. Sabin's laboratory, the solutions of dyes employed and their method of preparation will be described in detail.

We are now using six different solutions for our routine work. Solution 1 is prepared by dissolving 125 mg. of vital neutral red (Grübler) in 50 cc. of neutral absolute ethyl alcohol. This makes a 0.25 per cent solution which is saturated. It may be stated here that neutral red (Ehrlich) certified for use in vital staining as prepared by the National Aniline and Chemical Company, New York, is a thoroughly satisfactory dye which can be substituted for neutral red (Grübler). Solution 2 is prepared by dissolving 125 mg. of vital Janus green (National Aniline or Grübler) in 62.5 cc. of neutral absolute ethyl alcohol, thus making a 0.20 per cent solution which likewise is saturated. By adding 50 drops of Solution 1 to 10 cc. of neutral absolute ethyl alcohol, we prepare Solution 3. Solution 4, a mixture of neutral red and Janus green, is made by adding 2 drops of Solution 2 to 3 cc. of Solution 3.

For the study of blood or other tissues, where cells to be stained are relatively few in number, we prepare slides in the manner described by Sabin (5). This is accomplished as follows: (a) by means of a pipette, flood the upper surface of a slide chemically clean with Solution 4; (b) drain excess of dye back into bottle by holding slide in an upright position; (c) absorb the excess of dye on the dependent edge of the slide with blotting material; (d) evaporate the remaining alcohol on the slide by immediately holding it over a piece of wire gauze under which a gas flame is burning. There is no objection to burning the excess of alcohol from the slide. The stained surface of the slide is then marked with a wax pencil and the slides are stored for use. Some investigators in the past have experienced difficulties in obtaining an even distribution of the dye over the slide, particularly when there is a high degree of humidity in the atmosphere. Preparing the slides as described, in artificially dried air, obviates these troubles, leaving a thin, even film of the dye.

For study of bone marrow, lymph nodes, or other tissues where there is an abundance of colorless cells to be stained, two more solutions are required. Solution 5 is prepared by adding 150 drops of Solution 1 to 10 cc. of neutral absolute ethyl alcohol. Solution 6, a mixture of neutral red and Janus green, is made by adding 8 drops of Solution 2 to 3 cc. of Solution 5. Thus Solution 6 contains approximately three times the amount of the dyes as Solution 4. Slides are prepared with Solution 6 in precisely the same manner as with Solution 4. The latter solutions, 4 and 6, being mixtures of neutral red and Janus green, deteriorate after about 24 to 48 hours and must be freshly prepared each time they are needed. The remaining solutions are stable and are best kept in glass stoppered bottles in a cool, dark place. Care must be exercised to make sure that the pipettes and bottles used are free of alkali. If an excess of alkali is present, the neutral red solution will become a muddy yellow color.

Blood films in these supravital studies are prepared by obtaining a drop of blood on a clean coverslip and letting it fall gently on the slide prepared from Solution 4. The film is quickly rimmed with vaseline (salvoline) and in the course of from 3 to 5 minutes is ready for study. The films are best studied in a constant temperature box at 38° or 39°C. For the study of the cells of organs such as the omentum or subcutaneous tissues, thin films of the intact tissue may be spread over slides prepared with Solution 6. If the organs are too dense or too thick to be spread intact over a slide, the cut surface of the fresh tissue, for example, lung, lymph nodes, etc., is scraped with a sharp scapel and the tissue accumulating on the knife blade is placed on a slide prepared from Solution 6. If the tissue is very dry a drop of plain normal salt solution or normal salt solution moderately colored with neutral red may be added to suspend the cells. A coverslip is then applied as above and is rimmed with vaseline. Material prepared in this way provides a thin enough film so that the individual living cells can be studied. For our purposes, tissues fixed in Helley's fluid, embedded in paraffin, and stained by the usual methods were used to supplement the supravital studies.

The fundamental experiments dealing with giant cells were carried out on the blood and tissues of rabbits which had been injected with various substances, namely, agar, lycopodium spores, paraffin, olive oil, mineral oil, living tubercle bacilli, and certain lipoid fractions isolated from tubercle bacilli. These latter substances were obtained from Dr. R. J. Anderson of the Sterling Chemical Laboratories of Yale University, through the Research Committee of the National Tuberculosis Association. A full account of these reactions has been presented by Doan, Sabin and Forkner (1). None of the substances studied produced giant cells in the circulating blood, except agar. Consequently the development of this special phase of the giant cell study is here presented separately.

Experimental Data

The early experiments in which relatively small amounts of agar were introduced into the tissues produced no significant changes either quantitative or qualitative in the cells of the blood. However, since the local tissue response was so profound such large numbers of monocytes and giant cells having been produced, the possibility presented itself that a larger and more disseminated foreign body reaction might carry over the tissue response to the blood. This theory seemed to have merit since it was known from experience in this laboratory that certain diseases, notably tuberculosis, are often associated with the finding of pathologic tissue elements, epithelioid cells, in the circulating blood.

Accordingly, an animal, R 790, was injected, on February 25, 1929, with plain sterile agar in many areas under the skin and intraperitoneally.

A total of 40 cc. of the agar at pH 7.4 was introduced. Incidentally this animal was given frequent intravenous injections of trypan blue in order that the reactions of the giant cells of the tissues to this vital dye might be studied. Their responses are discussed elsewhere (1). Let it suffice to say that the trypan blue did not materially influence the blood picture in this animal, except that it may have been responsible for the increased number of macrophages.

The blood had been examined prior to the injections, but was not studied again until 1 week later (March 4). At this time there was a slight reduction of the hemoglobin and number of red corpuscles. The total white blood cell count was not changed. Monocytes were distinctly high (21 per cent) and the lymphocytes were lower than they had been in the control period. The blood platelets were clustered in large masses. Some individual blood plates were larger than normal. The platelets were undoubtedly greatly increased in number. The blood contained 11 per cent of clasmatocytes (macrophages) which were usually large cells with single, centrally placed nuclei. Their cytoplasm contained débris of various shades of color and various sizes and shapes. The presence of the large number of these cells on this occasion may be explained as the result of the injection of trypan blue. Such phenomena have been studied and reported by Simpson (8). The character of the monocytes on this and subsequent days was of great interest. Many of them were much larger than normal, possessed round or oval nuclei, often with two or three nucleoli, and each cell contained a rosette of exceedingly fine uniform neutral red bodies. In some instances the rosettes were small, whereas in others they were hypertrophied, making a stimulated monocyte which is a transition between a normal monocyte and an epithelioid cell. These forms are entirely similar to the developing monocytes which Forkner (27) has described and illustrated as normal elements in peripheral lymph nodes of rabbits. Another interesting change was noted in the polymorphonuclear neutrophils. Many of these cells had a decreased number of specific granules and contained more neutral red bodies than are normally present. These neutral red bodies normally are round or globular whereas after agar injections the same bodies are present and in addition many larger, often elongated, non-refractive vacuoles or granules.

Besides these changes in the usual blood cells, typical multinucleated giant cells could be found in every smear on this and subsequent days until the animal was autopsied on March 18, 1929, 3 weeks after the agar injection. The photograph of one of these cells is illustrated in Fig. 11. This cell had about 25 nuclei scattered through all parts of the cytoplasm. There was an area near the center of the cell which was probably agar. It was a homogeneous amorphous material which had a purple tint. It had apparently taken on some of the trypan blue as the agar masses in the tissue had done. The nuclei near the periphery tended to be oval, whereas the others were round in shape. Individual nuclei were quite uniform in size and were somewhat larger than red blood corpuscles. Some of them contained nucleoli. The cytoplasm contained scattered, irregular masses of material staining all shades of red, yellow, and brown. There was no pattern to the arrangement of the material stained with neutral red.

Fig. 9 represents a cell from the peripheral blood of this animal. A coarse mass of agar is seen to lie across the center of the cell and on either side are segregation bodies entirely similar to those encountered in epithelioid cells and monocytes. One can, in fact, make out two areas in which the arrangement of the vacuoles suggests rosettes of neutral red bodies in the upper part of this cell, indicating a possible fusion of two or more mononuclear or polynuclear cells around the large mass of agar. This last cell (Fig. 9) is difficult to classify in one or the other group, but on the whole it is more like a foreign body giant cell.

Another interesting group of cells from Rabbit R 790, shown in Fig. 4, illustrates how the giant cells may be formed or how they may be disintegrated. In this figure are three stimulated monocytes which have fused together about a blue mass of agar. When first seen this group had an almost spherical cell membrane surrounding the three cells and the agar. They were observed in the warm box for about 2 hours and were seen to separate into three distinct living cells with only a thread of cytoplasm connecting them. The agar was left free between them. This phenomenon may represent the fate of foreign body giant cells by disintegration into separate uninuclear cells. Other instances have been observed where giant cells of the foreign body type could be seen to be composed of many individual monocytes with other monocytes or epithelioid types nearby, apparently fusing to form a giant cell (Figs. 12 and 13). It would appear then that this is evidence similar to that recorded by Hektoen (13). He studied healing, nondegenerative tuberculous tissue of the brain and found that many giant cells are disintegrated into mononuclear cells which retain their function. It is not clear from his text whether the giant cells which he observed as separating into mononuclear elements were according to the classification here employed "epithelioid giant cells" or foreign body giant cells. I have found no evidence for this phenomenon in "epithelioid giant cells." Later Hektoen (28) supported his former view by observing the same phenomenon in the fate of the giant cells which form in the absorption of coagulated blood serum, in the anterior chamber of the rabbit's eye. In these latter experiments it seems that he was probably dealing with the foreign body type of giant cells.

Rabbit R 579 was injected with agar as the previous animal had been, but no dye was introduced.

A preliminary study of the blood before the injection showed a normal formula. There were 7,600 white blood cells and 5,250,000 red blood cells per cubic millimeter. There were 11 per cent monocytes. 2 days after the injection of agar, the monocytes rose to 20 per cent and increased in total number from 836 to 1,580 per cubic millimeter. 6 days after the injection, monocytes became more numerous than any other cell, increasing to 49 per cent or 5,120 cells per cubic millimeter. A chart (Chart 1) makes this reaction clear. In this experiment, there was a reciprocal relationship between the numbers of monocytes and lymphocytes. As in the preceding experiment, many of the monocytes were of the young type, larger than the adult cells, possessing round or oval nuclei with nucleoli and having an extraordinary abundance of delicate mitochondria. Some of the mature monocytes contained coarse irregular brownish bodies in their cytoplasm, which were probably pieces of agar undergoing digestion.

Giant cells were seen in the blood on the eighth and ninth days. On the eighth day enough were present to be recorded in the differential count on a percentage basis; on the ninth day, only an occasional cell in an entire film, indicated on the chart by a + sign, was seen. One of these was drawn (Fig. 6). It is clearly of the foreign body type with scattered nuclei and abundant irregular masses of material in the cytoplasm, probably agar undergoing fragmentation and disintegration. The nuclei contained definite nucleoli. Mitochondria were present and easily seen near the periphery of the cell. Adjoining this cell but not fused with it is a monocyte containing the same kind of material as in the giant cell. It is apparent that the larger cell might easily be considered as representing a fusion of many of the individual monocytes. Another cell was drawn (Fig. 7) which has a central rosette and three peripheral nuclei. The material in the cytoplasm is

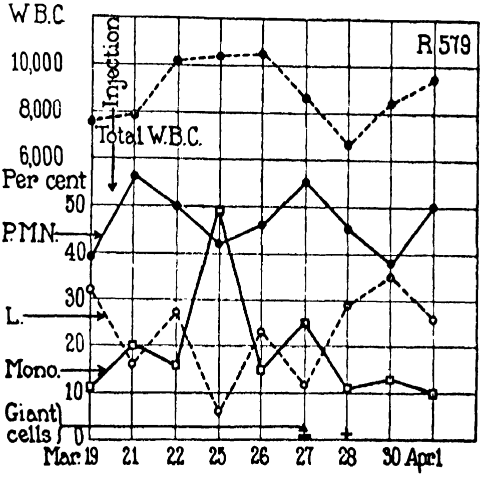

CHART 1. Graph of the white blood cells of Rabbit R 579. The arrow indicates the time at which 40 cc. of sterile plain agar was given in many subcutaneous areas and intraperitoneally. For giant cells the symbol + means the presence of an occasional giant cell in an entire preparation; the solid blocks indicate their percentages when sufficient to appear in the differential count.

not typical of an epithelioid cell. It looks much more like a small foreign body giant cell. It is impossible to say whether this giant cell has arisen by fusion of independent elements or has formed by division of the nuclei. Fig. 8 represents a small foreign body giant cell of the same type as Fig. 7, but containing much coarser bodies. The nuclei are flattened out against the cell wall. This cell, together with that shown in Fig. 7, illustrates that one can not always identify this type of giant cell by the peripheral arrangement of the nuclei. In order to differentiate them properly one must study all of the cellular organs. The presence of a central rosette of fine neutral red bodies aids materially and is probably the most important single factor in the separation of "epithelioid giant cells" or the so-called Langhans' cell. The lungs of this and the other animals were studied in

TABLE I

Changes in the Blood Cells as the Result of Agar Injections into the Tissues

Experiment	Date	White blood cells per cu. mm.	Red blood cells per cu. mm.	Neutrophils		Basophils		Eosinophils		Monocytes		Lymphocytes		Giant cells		Clasmatocytes		Unclassified		Hemoglobin per cent
				Per cent	Per cu. mm.	Per cent	Per cu. mm.	Per cent	Per cu. mm.	Per cent	Per cu. mm.	Per cent	Per cu. mm.	Per cent	Per cu. mm.	Per cent	Per cu. mm.	Per cent	Per cu. mm.	
R 790	1/12/29	4,850	5,490,000	45	2,182	5	242	1	48	11	533	38	1,843							68
	1/14	6,400	5,800,000	30	1,920	14	896	2	128	7	448	47	3,008							65
	2/25	6,250	6,200,000	56	3,500	8	500	1	63	7	438	28	1,750							76
	2/25	Began frequent intravenous injections of trypan blue. Injected 40 cc. of agar intraperitoneally and subcutaneously.																		
	3/4	5,500	4,580,000	52	2,860	6	330			21	1,155	10	550		+	11	605			57
	3/6	4,100	4,000,000	58	2,378	14	574	1	41	6	246	18	738		+	3	123			56
	3/7	7,500	4,140,000	63	4,725	10	750	1	75	2	150	22	1,650		+	1	75	1	75	55
	3/9	3,800	4,360,000	62	2,356	12	456	1	38	5	190	19	722		++					49
	3/11	4,200	4,940,000	72	3,024	1	42	2	84	1	42	23	966		+	1	42			56
	3/12	7,950	4,480,000	72	5,724	2	159	2	159	3	238	21	1,669		+					61
	3/15	8,300	3,790,000	68	5,644	18	1,494	1	83	4	732	8	664		+	1	83			53
	3/18	7,250	4,690,000	53	3,842	22	1,595			8	580	13	942		+	2	145	2	145	62
R 579	3/19	7,600	5,260,000	39	2,964	17	1,292	1	76	11	836	32	2,432							
	3/19	Injected in 6 subcutaneous areas intraperitoneally with 40 cc. of plain agar.																		
	3/21	7,900	4,840,000	56	4,424	5	395			20	1,580	16	1,264					3	237	52
	3/22	10,200	4,540,000	50	5,100	4	408	2	204	16	1,632	27	2,754					1	102	55
	3/25	10,450	5,260,000	42	4,389	1	104			49	5,120	6	627					2	209	64
	3/26	10,550	5,210,000	46	4,853	10	1,055	4	422	15	1,582	23	2,426					2	211	59
	3/27	8,700	5,320,000	55	4,785	3	261			25	2,175	12	1,044	1	87			4	348	64
	3/28	6,700	4,620,000	45	3,015	12	804	2	134	11	737	29	1,943	++	++			1	67	58
	3/30	8,450	4,520,000	38	3,211	13	1,098	1	85	13	1,098	35	2,957							
	4/1	9,500	4,790,000	50	4,750	12	1,140	2	190	10	950	26	2,470							55

R 580	3/19	11,000	4,280,000	37	4,070	9	990	1	110	9	990	43	4,730					1	110	60
	3/19	4 cc. trypan blue every 2nd day and 40 cc. plain agar injected intraperitoneally and subcutaneously today.																		
	3/21	45,500	4,440,000	52	23,608	4	1,816			37.5	17,025	4.5	2,043		+			2	908	52
	3/22	21,900	4,630,000	53	11,607	1	219			40	8,760	4	1,314							65
	3/23	42,850	4,300,000	41	17,569	1	429			38	16,283	11	5,994		+	1	429	5	2,142	
	3/25	19,050	3,920,000	49	8,954	2	381	2	381	28	5,905	8	1,524		+	1	191	9	1,715	55
	3/26	24,950	4,170,000	62	15,469	3	749			11	2,745	8	2,745		+			13	3,244	58
	3/27	5,400	4,520,000	56	3,024	9	486	4	216	14	864	15	810		+					60
	3/28	4,400	4,320,000	55	2,420	7	308			14	660	20	968	1	44					52
R 967	3/16	Injected intravenously with 1 mg. T. B.																		
	3/25	5,300	6,150,000	52	2,756	8	424	1	53	4	212	34	1,802					1	53	62
	3/28	Injected subcutaneously and intraperitoneally with 40 cc. trypan blue every 2nd day.																		
	3/29	5,050	5,270,000	77	3,889	7	353			6	303	8	404							52
	3/30	6,150	4,780,000	42	2,583	3	185			42	2,583	9	553	+	+			4	246	54
	4/1	5,400	5,240,000	29	1,566	5	270	1	54	41	2,214	15	810	4	216	5	270			52
	4/3	7,350	5,850,000	24	1,764	11	808			39	2,866	19	1,397		++	3	220	4	294	54
	4/5	5,000	5,060,000	34	1,700	9	450			34	1,700	14	700	5	250			4	200	62
	4/6	5,150	5,110,000	65	3,347	8	412			17	875	10	515							54
	4/8	6,400	5,520,000	51	3,264	6	384	1	64	12	768	17	1,088	7	448	1	64	5	320	56
	4/9	5,800	5,260,000	61	3,538	5	290			13	754	19	1,102	2	116					56
	4/10	18,300	4,820,000	71	14,823	1	183			14	2,562	4	732		++					64
	4/11	5,950	5,260,000	64	3,808	3	178			11	655	14	833	3	179	4	238	1	60	53
	4/12	3,850	5,280,000	40	1,540	6	231			33	1,270	7	270	3	115	5	193	6	231	55
	4/13	12,500	5,020,000	77	9,625	4	500			9	1,125	7	875	1	125	1	125	1	125	52
	4/15	8,950	4,790,000	81	7,339	3	269			9	806	3	269	2	179	1	89			52
	4/16	6,850	4,020,000	77	5,275	5	342			10	685	5	342	1	69	1	69	1	69	44

TABLE I—*Concluded*

Experiment	Date	White blood cells per cu. mm.	Red blood cells per cu. mm.	Neutrophils		Basophils		Eosinophils		Monocytes		Lymphocytes		Giant cells		Clasmatocytes		Unclassified		Hemoglobin per cent
				Per cent	Per cu. mm.	Per cent	Per cu. mm.	Per cent	Per cu. mm.	Per cent	Per cu. mm.	Per cent	Per cu. mm.	Per cent	Per cu. mm.	Per cent	Per cu. mm.	Per cent	Per cu. mm.	
R 939	5/20	8,450	4,610,000	49	4,140	4	338			23	1,943	22	1,859					2	169	
	5/20	Injected intraperitoneally and subcutaneously with about 40 cc. sterile plain agar containing 10 cc. of India ink.																		
	5/21	26,100	3,690,000	76	19,836	2	522	3	783	14	3,654	5	1,305							
	5/22	18,850	3,810,000	60	11,310					25	4,712	14	2,639		+			1	188	
	5/23	10,300	4,490,000	65	6,695	1	103			27	2,781	7	721							
	5/24	14,850	4.680,000	50	7,425	4	594			23	3,415	19	2,821	3	445			1	148	
	5/27	6,750	4,620,000	47	3,172	3	202			24	1,620	22	1,485	4	270					
	5/29	7,650	4,330,000	67	5,125	2	153			11	841	20	1,530		+					
	5/31	6,500	4,640,000	65	4,225	2	130	3	195	8	520	22	1,430		++					
	6/3	10,100	4,910,000	80	8,080	1	101			2	202	16	1,616		+			1	101	
	6/5	8,750	4,200,000	92	8,050					4	350	3	262					1	87	
	6/6	8,150	4,930,000	86	7,009	8	652			2	163	4	326							

sections. Numerous giant cells were easily seen in the smaller capillaries. Fig. 14 illustrates one of the giant cells in the lung of this animal.

Rabbit R 580 received the same type of injections as R 790.

Trypan blue (1 per cent solution in distilled water) was given intravenously or intraperitoneally in 4 to 10 cc. doses every second day and in addition the animal received plain agar into many areas of the subcutaneous tissue and peritoneal cavity. Prior to the injections the cells of the blood were entirely normal (Table I, Chart 2). There were 11,000 leucocytes per cubic millimeter. However, on the day following, the total leucocyte count rose to 45,500 cells per cubic millimeter—an increase of over 400 per cent. This rise was chiefly brought about by the monocytic strain of cells. The neutrophiles also increased somewhat, but the lymphocyte curve descended, their number being represented by only 4 per cent of the total white

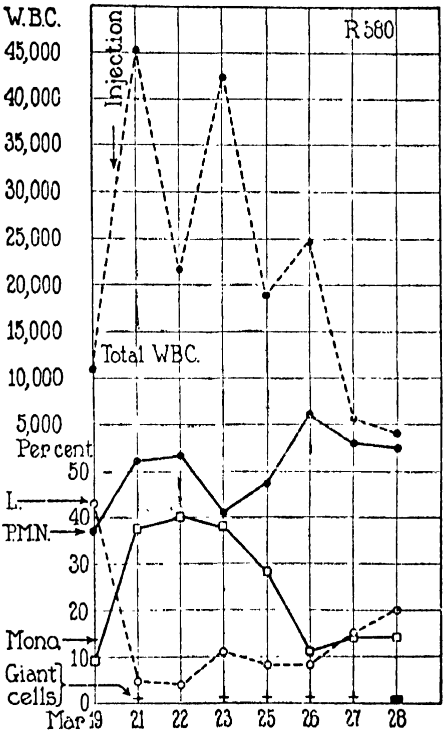

CHART 2. Graph of the white blood cells of Rabbit R 580. The arrow indicates the time at which 40 cc. of sterile plain agar was introduced subcutaneously and intraperitoneally. At this point injections of trypan blue were begun and were continued on every second day.

blood cells. At the height of the reaction there were over 17,000 monocytes in each cubic millimeter of blood. Many of these monocytes were of the young type previously described. Some of the monocytes and neutrophiles appeared to contain fragments of agar. Many of the neutrophiles had lost a considerable portion of their specific granules. There was a tendency for the leucocytes to be clumped in large masses. Some of the young monocytes contained two oval nuclei.

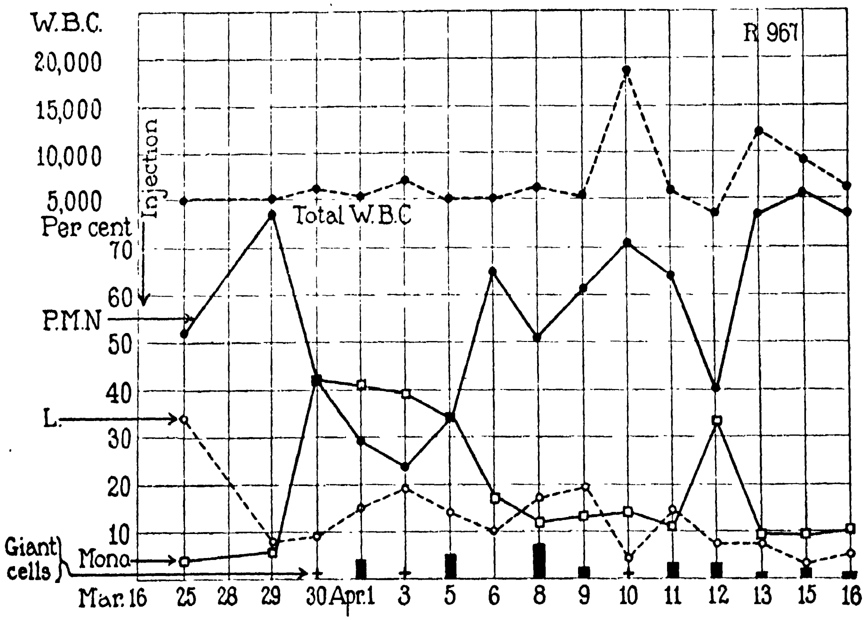

CHART 3. Graph of the white blood cells of Rabbit R 967. The first arrow shows the time at which the rabbit received 1 mg. of avirulent tubercle bacilli intravenously. This presumably did not modify any of the changes found in the chart. The second arrow indicates the time at which 40 cc. of sterile plain agar was administered subcutaneously and intraperitoneally. At this time also injections of trypan blue were begun and they were continued every second day.

2 days after the agar injection, the total leucocyte count was 21,900 per cubic millimeter, but the percentage of monocytes was even higher (40 per cent). The accompanying table and chart (Table I, Chart 2) will make these changes clear. 3 days after the beginning of the experiment the total white blood cell count was again elevated to 42,850 cells per cubic millimeter, but there were no significant changes in the percentages of cells except a rise of lymphocytes from 4 to 11 per cent. Giant cells of both types began to appear in the blood and could be found in practically every smear thereafter. 6 days after the injection, a mild leucopenia set in with the monocytes constituting about 14 per cent of the cells.

Rabbit R 967 was interesting because it showed giant cells in large numbers in the blood stream.

On March 16, 1928, the animal received an intravenous injection of 1 mg. of avirulent tubercle bacilli which did not produce progressive tuberculosis and which presumably caused no significant changes in the blood picture to be described. The blood picture was normal on March 25 when intravenous injections of trypan blue were begun and continued every second day for nine doses. This may account for the presence from time to time of from 3 to 5 per cent of clasmatocytes (macrophages) in the blood. The animal was injected with plain agar in a number of areas on March 28. The chart and table (Table I, Chart 3) show the changes in the total number and percentage values of the cells. The chief result of the injection on the blood was a rather striking and prolonged rise in monocytes together with a maintained decrease in the percentage and absolute number of lymphocytes.

This animal differed from the preceding one in that there was no sharp rise in total leucocytes within a short time after the injection. However, there did occur the marked elevation of percentage of monocytes. This increase was found on the second day and persisted for 1 week during which time the monocytes ranged from 34 to 42 per cent of the circulating white blood cells. During the next 2 weeks monocytes remained abundant, varying between 9 and 33 per cent. Giant cells occurred in the blood 2 days after the agar injection and were found in every film of blood until autopsy 17 days later. They frequently were so numerous as to constitute 2 or 3 per cent of the total number of leucocytes and on one occasion reached as high as 7 per cent. It was not uncommon to find two in a single high power field of the microscope. Most of the "epithelioid giant cells" contained from 2 to 7 nuclei (Fig. 15) but an occasional one had from 10 to 14 nuclei; the foreign body giant cells, on the other hand, contained from 2 to 50 nuclei, many of them having from 15 to 30.

Fig. 2 illustrates what might be called a mononuclear giant cell of the blood. It measured 26.7 by 31.4 microns, about twice the size of an ordinary monocyte. It had a large indented nucleus with a nucleolus, a definite rosette of fine neutral red bodies, together with numberlesss delicate mitochondria. This cell would fall into the monocyte group and would be further qualified by saying that it is a young stimulated form. If the rosette were slightly larger we would call it an epithelioid cell. 4 days after the injection, when monocytes constituted 41 per cent of the white blood cells, this type of cell, the young, large, stimulated form, was very abundant. Cells entirely identical with this except for the presence of 2, 3, or more nuclei were frequently found. Fig. 3 illustrates one of these epithelioid giant cells with 9 nuclei. All intermediate stages between the cells represented by Figs. 2 and 3 could readily be demonstrated in the blood.

At first the mechanism by which the giant cells reached the peripheral veins was not understood for some of these cells were more than

50 microns in their smallest diameters. It is difficult to conceive how these cells could find their way through the capillaries of the lungs and then into the systemic circulation. Figs. 5 and 10 illustrate how this phenomenon is accomplished. The cell in Fig. 10 was taken from the peripheral ear vein of Rabbit R 967 and shows that these giant cells are not rigid but that they may become elongated to such an extent as to pass through very small capillaries. Fig. 5 was from the central artery of the ear of Rabbit R 939 and illustrates the apparent ease with which the cells may make their way through the capillary bed.

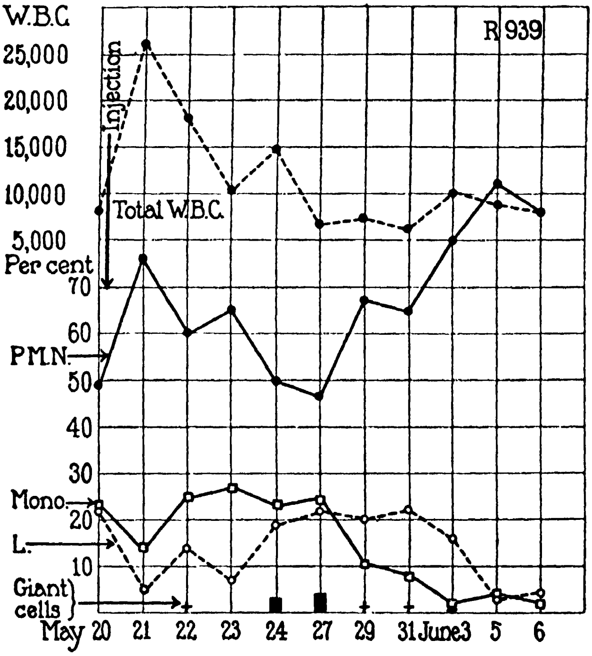

CHART 4. Graph of the white blood cells of Rabbit R 939. The arrow indicates the time at which 40 cc. of sterile plain agar was injected into the subcutaneous tissues and into the peritoneal cavity.

The next experiment which was tried was to mark the agar in some way in order that it might be identified when it was transported within cells and to distant parts of the body. This was carried out by adding to the 40 cc. of agar to be injected 10 cc. of Higgins' India ink. This was mixed carefully and made an intensely black preparation.

Rabbit R 939 was chosen for the experiment. The count prior to the injection showed the animal to have 23 per cent, an abnormally high value, of monocytes (Table I, Chart 4). On the day following the injection, the total leucocytes rose to 26,100 cells per cubic millimeter. The monocytes rose and remained high for several days. Giant cells of both types appeared in very small numbers in the blood on the second day and were found on several occasions over a period of 2 weeks. Occasionally a few granules of carbon could be seen in the giant cells, but not as much as was expected. Fig. 1 shows an epithelioid giant cell taken from the arterial blood, which has several definite fragments of agar containing carbon. The autopsy on this animal demonstrated that the carbon had not been as evenly distributed in the agar as was expected. The giant cells immediately adjacent to the ink deposits contained it in great abundance, but most held relatively little.

Many more animals were subjected to similar experiments with agar and with other substances. It was not possible to induce regularly an appearance of cells in the blood with any of the substances used except agar. In one animal which had been injected with large amounts of lycopodium spores, one giant cell was found in the blood on a single occasion. This finding could not be repeated.

DISCUSSION

These studies on the peripheral blood have made it possible to form definite ideas about the origin and development of "epithelioid giant cells" and of foreign body giant cells. It becomes necessary to consider these elements histologically as two groups of cells which have a common origin from monocytes. Under the conditions of these experiments it appears that the great majority of foreign body giant cells are formed by the fusion of monocytes, and that the majority of "epithelioid giant cells" are formed by amitotic division of the nucleus or nuclei of epithelioid cells which latter elements are monocytes of large size, with hypertrophied rosettes. The results of the studies would thus confirm the theories of both schools of pathologists, of those who consider that giant cells are the products of fusion, and those who maintain that they form by division of the nuclei. The difference of opinion on this point has resulted doubtless from the failure to recognize that in almost every tissue reaction which produces giant cells, both types are present, but that their relative numbers assume different proportions according to the character of the substances which excite the response. No evidence has been observed in these studies to support the theory that epithelioid giant cells are formed by the fusion of individual cells. On the other hand, many epithelioid cells and "epithelioid giant cells" with partially divided nuclei, and others which appeared as though the nuclei had recently divided amitotically have been seen.

Regarding the fate of giant cells, no definite conclusions are possible but these studies lend support to the conclusion of Hektoen (28) that foreign body giant cells may separate into their constituent elements. No evidence is available that "epithelioid giant cells" undergo a similar process.

The occurrenceof the two types of giant cells in the circulating blood may be explained in either of two ways. They may be formed in the blood vessels in the neighborhood of the foreign body reaction, or they may form extravascularly and migrate into the vessels. The latter explanation would appear more tenable, for giant cells are to some extent motile, and can become so elongated that passage through the capillary walls should be entirely possible. Furthermore, it would be difficult to explain the presence of large pieces of agar in the cells, as shown in Figs. 4 and 9, by any other hypothesis than that they have been carried within cells from the tissues into the blood.

SUMMARY AND CONCLUSIONS

1. It has been demonstrated that giant cells of the foreign body and epithelioid types can be induced to appear in the blood stream.

2. Evidence has been presented which indicates that foreign body giant cells are primarily formed by fusion of monocytes and that the fate of these giant cells is accomplished, at least in some instances, by a separation into the constituent elements.

3. Further evidence has been presented which lends support to the hypothesis that "epithelioid giant cells" reach their stage of evolution, not by fusion of monocytes, but by amitotic division of the nuclei of monocytes and epithelioid cells.

4. The presence of giant cells in the peripheral blood as the result of agar injections is almost invariably associated with, or preceded by a marked monocytosis in which the new monocytes are of large size and show evidence of immaturity.

5. Injections of agar into the tissues result in decreased absolute and percentage values of lymphocytes and a diminution of the specific granules in many of the polymorphonuclear leucocytes.

6. It would appear from these studies that a clear differentiation of "epithelioid giant cells" and "foreign body giant cells" in the blood is usually possible, but that on the other hand, a few cells may be present which have some of the characteristics of each type. These latter cells probably represent in their formation both a fusion of individual cells and an amitotic division of the nuclei of monocytes.

7. Clasmatocytes or macrophages have in rare instances been seen to take part in the formation of foreign body giant cells. At least one instance has been noted of the fusion of a clasmatocyte with several monocytes. No evidence is available to demonstrate that macrophages ever play a part in the formation of "epithelioid giant cells."

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EXPLANATION OF PLATES

PLATE 10

Drawings of cells from arterial and venous blood of rabbits after injections of agar; stained with neutral red and Janus green and drawn while still alive.

FIG. 1. An epithelioid giant cell from the peripheral arterial blood of Rabbit R 939. Small aggregations of carbon can be seen. The mitochondria are very abundant and are best seen at the periphery of the rosette. This cell measured 39.3 x 33 microns.

FIG. 2. A huge, young, stimulated monocyte from Rabbit R 967. It is almost as large as the cell in Fig. 1, but contains only a single nucleus with a large nucleolus. Thousands of delicate mitochondria are stained with Janus green and a typical dense rosette is stained with neutral red. Cells identical with this, except for the presence of two or more nuclei, could be found in practically every field. This cell measured 31.4 x 26.7 microns.

FIG. 3. Epithelioid giant cell from venous blood of Rabbit R 967, with nine nuclei. It has a unified central rosette, similar to those of Figs. 1 and 2. Size 36.1 x 33 microns.

FIG. 4. Three monocytes which have fused around a piece of agar in the blood of Rabbit R 790. This animal had received trypan blue intravenously on several occasions and the agar foci were stained in an identical manner, as illustrated in this figure. When first seen this cell was practically spherical, but after 2 hours the three constituent cells were almost entirely independent of each other. Size 35 microns at widest diameter.

FIG. 5. This huge giant cell was obtained from a peripheral artery of Rabbit R 939. It measured 107 microns in length. It illustrates very well that these cells are capable of changes in shape so great as to enable them to pass through the capillaries. A red blood corpuscle is shown to give an idea of their great sizes.

FIG. 6. A foreign body giant cell with many nuclei from the venous blood of Rabbit R 579. This cell and the monocyte nearby are filled with masses of irregular, fragmented material. This material is probably agar undergoing changes within the cell. Size 67.5 × 47.1 microns.

FIG. 7. A trinucleated cell containing the same general type of material as that in the cell of Fig. 6. The mitochondria have faded somewhat. This cell is also from the blood of Rabbit R 579. Size 21 microns in diameter.

FIG. 8. Another foreign body giant cell from the blood of the same animal. Size 28 × 17 microns.

FIG. 9. A foreign giant cell from the blood of Rabbit R 790. There is a large, lobulated mass of material in the center of the cell which is probably agar that has not as yet undergone very much change within the cell. In the upper left region of the cell two rosettes can be seen. Size 84 x 52.5 microns.

FIG. 10. Another elongated cell. This foreign body giant cell was from the blood of Rabbit R 967. Size 73.8 by 14 microns.

PLATE 11

Four photographs of living cells supravitally stained with neutral red and Janus green, and one photograph (Fig. 14) from a section.

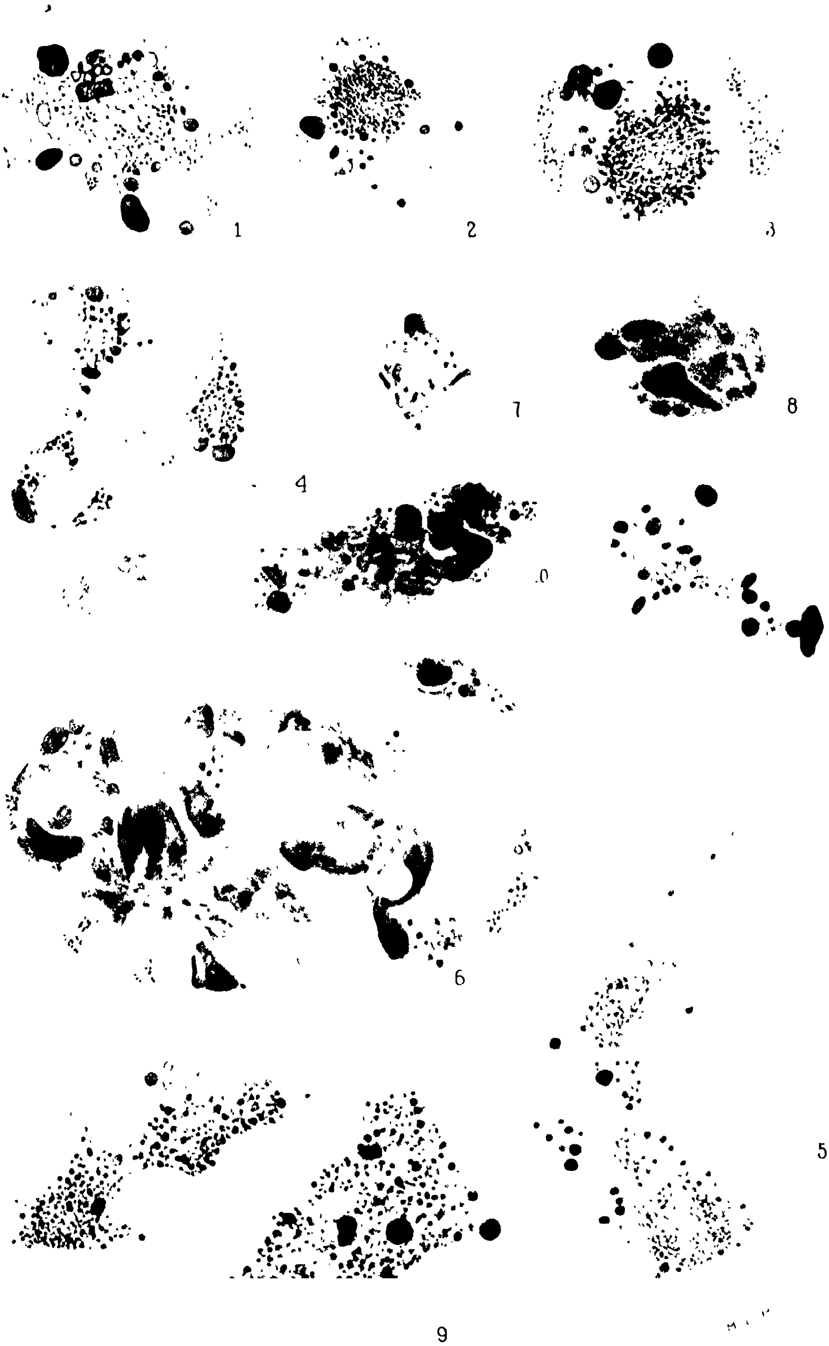
FIG. 11. Photograph of a large foreign body giant cell from the blood of Rabbit R 790. It has very many nuclei scattered throughout the cell and the cytoplasmic bodies are arranged in no definite pattern. Several monocytes can be seen on the surface of the cell, as if in process of fusion. Red blood corpuscles can be seen for comparison of size. Magnification × 575. Stained with neutral red in supravital preparation.

FIG. 12. Photograph of a foreign body giant cell in the blood with many monocytes and epithelioid cells about it. This animal, R 939, had received agar plus India ink which was injected under the skin and into the peritoneal cavity. Note the definite rosettes of neutral red bodies in the monocytes and epithelioid cells. Magnification × 750. Stained with neutral red in supravital film.

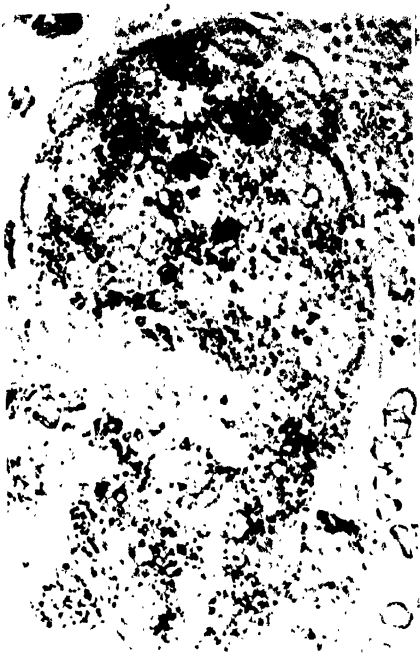
FIG. 13. Photograph of an accumulation of monocytes about a foreign body in the blood of Rabbit R 967. The cell at the upper end of the figure is a clasmatocyte. The cytoplasm of these cells could not be separated. They were apparently fused. A single monocyte may be seen nearby at the lower border of the figure. Magnification × 750. Stained with neutral red by means of the supravital method.

FIG. 14. Photograph of a giant cell in a capillary of the lung of Rabbit R 579. Many giant cells could be demonstrated in the lungs of this and other animals similarly treated. Magnification × 750. Stained in fixed section with hemotoxylin and eosin.

FIG. 15. Photograph of an epithelioid giant cell in the blood of Rabbit R 967. Note the unified central rosette and peripheral nuclei. Contrast with Fig. 11. Magnification × 750. Stained with neutral red.



(Forkner: Multinucleated giant cells in blood)



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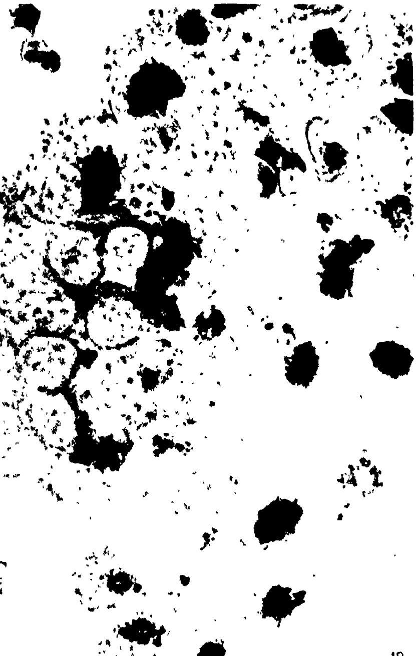

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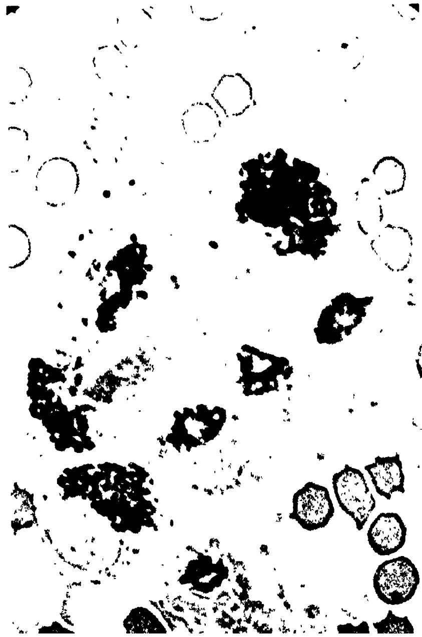

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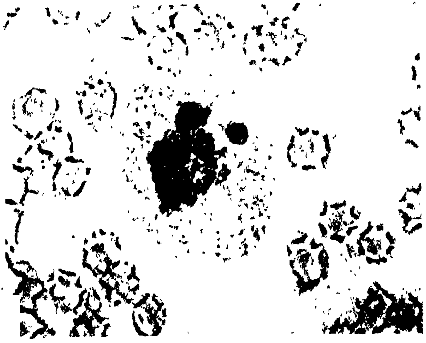

(Forkner: Multinucleated giant cells in blood)

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THE EFFECT OF CATHODE RAYS UPON CERTAIN BACTERIA

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PLATE 30

(Received for publication, March 26, 1930)

A study of the rate of killing of bacteria by cathode rays is a step towards finding out what changes these radiations and X-rays bring about in cells. The great interest to biology of X-rays, and probably also of cathode rays, lies not in the fact that they are lethal, but rather in their ability to produce permanent and inheritable changes in cell properties. Nevertheless, because they are deadly it is obvious that an understanding of the conditions under which cells are killed is a necessary preliminary to any thorough-going study of how they are changed. Bacteria have been used in the following experiments partly because they are, on the whole, the simplest cells to work with, partly because much has already been done on the lethal action of X-rays upon them, and partly because through their small size they provide the best introduction to the examination of the effects of these radiations upon the still smaller and imperfectly understood viruses.

Experimental Procedure

In the experiments to be described in this paper, single bacteria spread upon an agar plate are bombarded with a known number of cathode rays and, after incubation, the number of survivors is determined through counts of the colonies of bacteria. From these data and various well known physical characteristics both of the rays and of the bacteria, a satisfactory picture can be gained of many details of the destruction of these cells.

The cathode rays were obtained from a Coolidge type electron tube[1] leased from the General Electric Company. This tube is essentially a large hot cathode X-ray tube with a hollow anode covered by thin metal foil. Many electrons proceeding from the cathode pass through this anode and foil and, emerging into the air, constitute the cathode ray beam. The velocity of these electrons and, in general, the transparency of matter to them increases with the voltage applied to the tube. For these experiments the voltage was approximately 155 K.V. The velocity of the emitted electrons is of the order of 0.8 of the velocity of light.

The absorption of an electron in inanimate matter is attended by the release of a large number of ions within a very small volume. A 150 K.V. electron will liberate about 10^4 ions within less than 0.001 mm.[3] It is natural and in accord with existing knowledge to associate the changes brought about in a bacterium by cathode rays with the absorption of electrons and the consequent shower of ions freed within it. Together with this ionic shower, X-rays are emitted as a consequence of electron absorption. The passage of cathode rays through air is likewise attended by the formation of much ozone and it might be argued that the observed destructive action on bacteria was due either to these X-rays or to ozone. We have ascertained by suitable experiments that this is not the case.

Several conditions must be fulfilled if a statistical analysis of the killing of bacteria by cathode rays is to have any physical significance. In the first place, the absorption of these rays is so great that the bacteria must be exposed upon the surface of an agar plate rather than held in a suspension if the dose they receive is to be satisfactorily measured. It is likewise necessary that they be spread upon this surface with sufficient uniformity so that colony counts in selected standard areas can be taken as measures of the numbers of irradiated bacteria. Not only must the bacteria be spread as single organisms, but their multiplication must be prevented until irradiation is completed. This is evident when it is realized that if several organisms are associated together in a clump, a single survivor will produce a countable colony. Many experimental procedures were tried with several types of bacteria before one was found which gave a satisfactory spread of single organisms. The highly motile *B. aertryke* gave excellent results with 200 to 300 organisms per square inch and almost as satisfactory data were provided by *B. coli* in somewhat greater dilution. Repeated trials, however, failed to produce as good spreads of single organisms of *Staphylococcus aureus*. This bacterium has frequently been used in studies of the action of X- and ultraviolet rays. Our experience makes it seem probable that single cell spreads were not ordinarily obtained in these experiments.

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[1] Coolidge, W. D., *J. Franklin Inst.*, 1926, **202**, 693.

The bacteria were prepared for irradiation in the following way. Broth cultures, from standards provided by Dr. L. T. Webster of this Institute, were carried for at least 3 days through daily transplants before use. The final 20-hour old tube was diluted an hour before irradiation using either physiological salt or Locke's solution. This dilute suspension, so chosen as to give the desired number of bacteria per plate, was kept in an ice bath until actually used in order to prevent cell multiplication. In seeding, 1 cc. of this suspension was run evenly over the agar surface of a poured 10 cm. Petri dish. After vigorous shaking to remove the excess fluid, the plate was allowed to drain 10 minutes and its edges wiped free from adhering liquid. A known area was then immediately irradiated and another marked as standard. After incubation over night, counts were made of the colonies developing in each region. Approximately 100 plates were irradiated for each experiment. In some experiments the plates themselves were refrigerated before being inoculated. This proved to be unnecessary and had the disadvantage that the attendant condensation of moisture on the agar surface often destroyed the bacterial distribution.

TABLE I

Survival Ratios in Typical Experiments

Time	Survival ratios				
	(1) *B. coli*	(2) *B. aert.*	(3) *B. aert.*	(4) *B. aert.*	(5) *S. aureus*
4 sec.	0.698	0.562	—	—	0.853
5 "	.610	.417	0.699	0.671	.745
8 "	.455	.310	.602	.612	.566
12 "	.311	.258	—	—	.341
14 "	—	—	.481	.372	—
16 "	.204	.174	—	—	.248
20 "	.061	.151	.321	.264	.169
24 "	.050	.103	—	—	.168
28 "	.026	.055	—	—	.205
Electrons incident per cm.^{2}/sec.	1.65×10^9	—	0.85×10^9	0.96×10^9	2.99×10^9

The plate ready for irradiation was placed in the holder C of Fig. 1 and its agar surface pressed against the cutting edge of the tube A. This tube is divided lengthwise into two hemicylinders by a brass and lead partition. The electrons stream through the lower of these halves from the tube window, D, and strike the surface of the agar pressed into the cutting edge. The upper half, sealed with lead to exclude the electrons, provides a standard adjacent to the irradiated surface and of equal area. Cutting through the agar with the tube A has the double advantage of defining sharply the irradiated area and of marking permanently both it and the standard. The tube end was sterilized between irradiations by wiping with alcohol. The time of irradiation was controlled by operating a heavy lead shutter shown at B. In counting the number of electrons emerging from the tube, the measuring device to be described later replaced the plate, other conditions being the same as during irradiation.

In each experiment involving about 100 plates eight different times of exposure were commonly used and 10 or more plates were irradiated for each of these times. The ratios of the colony counts made after incubation upon their irradiated and standard areas were averaged for each time of exposure to give the typical results of Table I.

Analysis

These data can be analyzed with the help of the following considerations from elementary probability theory. If a' is the probability that an event will take place, by definition the probability of its not happening is $b' = 1 - a'$. Furthermore, if c' is the probability that some other event will occur, then the probability that a' and c' will happen together is $a'c'$. In the experiments of this paper many electrons are being shot at a few bacteria. If a is the probability that an electron will hit a bacterium and n is the number of electrons shot at it, the probability that one of these electrons will hit and that every other electron will miss it is

$$a\,(1 - a)^{n-1}$$

Since any one of the n electrons may hit, the probability of striking a bombarded bacterium only once is

$$P_1 = na\,(1 - a)^{n-1}$$

Similarly, the probability of hitting a bacterium by each of two selected electrons is a^2 and the probability of doing it only twice and by these two out of the n electrons is

$$a^2(1 - a)^{n-2}$$

If there are ${}_nC_2$ ways of combining two out of n electrons, the probability that a bacterium is hit twice and only twice is

$$P_2 = {}_nC_2\, a^2\,(1 - a)^{n-2} = \frac{n\,(n - 1)}{|\underline{2}}\, a^2\,(1 - \;)a^{n-2}$$

In the same way, the probability that a bacterium will be struck r out of n possible times is

$$P_r = {}_nC_r\, a^r (1 - a)^{n-r} = \frac{n(n-1)(n-2)\ldots\ldots(n-r+1)}{\underline{|r}}\, a^r (1-a)^{n-r}$$

The number of electrons striking the irradiated surface per second is of the order of 10^{10}. The number n, therefore, may be expected to be very large compared to r, the number of hits. If this is true, then

$$(1-a)^{n-r} \overset{\text{ca}}{=} (1-a)^n$$

and

$$n(n-1)(n-2)\ldots\ldots(n-r+1) \overset{\text{ca}}{=} n^r$$

and

$$P_r \overset{\text{ca}}{=} \frac{1}{\underline{|r}}\, a^r n^r (1-a)^n$$

Expanding $(1\text{-}a)^n$ by the binomial theorem gives

$$(1-a)^n = 1 - an + \frac{n(n-1)}{\underline{|2}}\, a^2 - \frac{n(n-1)(n-2)}{\underline{|3}}\, a^3 + \ldots\ldots\ldots$$

$$\overset{\text{ca}}{=} 1 - an + \frac{1}{\underline{|2}}\,(an)^2 - \frac{1}{\underline{|3}}\,(an)^3 + \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots$$

By MacLaurin's formula this is

$$(1-a)^n \overset{\text{ca}}{=} e^{-an}$$

In this way we arrive at the Poisson law[2]

$$P_r = \frac{1}{\underline{|r}}\,(an)^r e^{-an} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

in which an, the average number of hits, can be calculated from the results of irradiation. If one hit is enough to kill a bacterium, the survival ratios as given in Table I will be expressed by the probability that a bacterium will be missed every time. As the foregoing considerations have shown, this is

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[2] This expression or its equivalent has been used several times in studies of the effects of X-rays on cells. See for instance Condon, E. U., and Terrill, H. M., *J. Cancer Res.*, 1927, **11**, 324; Crowther. J. A., *Proc. Roy. Soc.*, 1926, B **100**, 390; Holweck, F., *Compt. rend.*, 1929, **188**, 197.

$$(1 - a)^n \overset{\text{ca}}{=} e^{-an}$$

In other words

$$\frac{A_1}{A_0} = \text{survival ratio} = e^{-an} \qquad (2)$$

If a bacterium could withstand one electron but were destroyed by the second, the survivors would be those which either escaped or were hit once, so that

$$\frac{A_2}{A_0} = e^{-an} + an\, e^{-an} = e^{-an}\,(1 + an) \qquad (3)$$

Similarly, if r hits are needed

$$\frac{A_r}{A_0} = e^{-an} + an\, e^{-an} + \frac{1}{2}\,(an)^2\, e^{-an} + \ldots\ldots\ldots\ldots$$

$$\ldots\ldots \frac{1}{\underline{|r-1}}\,(an)^{r-1}\, e^{-an}$$

$$= e^{-an}\left[1 + an + \frac{1}{2}\,(an)^2 + \ldots\ldots \frac{1}{\underline{|r-1}}\,(an)^{r-1}\right]$$

Standardization

The average number of hits can readily be calculated from a knowledge of (1) the area of the irradiated surface, (2) the dimensions of a bacterium, (3) the number of electrons striking the irradiated surface and (4) the absorption coefficient of electrons in the bacterium.

The counting of the number of electrons under the conditions of these experiments cannot accurately be carried out.[3] This is due partly to the enormous ionization produced in the air through which a cathode ray beam passes, partly to the fact that the absorption of the radiation from the tube in small thicknesses of matter is not linear and is accompanied by a considerable diffusion of the transmitted beam. Reproducible results which seem to be fairly accurate have, however, been obtained by the use of a counting chamber which consists of a brass disk enclosed, except for a $\frac{5}{16}$ inch hole on one side, by a grounded and air-tight brass sheath. This sheath is a cylinder 2 inches in diameter and 1 inch high and is provided with a port for evacuation. The central disk is supported by a thin rod which, mounted in amber, provides electrical connection with one of the galvanometer leads. The current produced by the flow of electrons from the collecting plate to ground through a sensitive galvanometer has been taken to measure the number of electrons in the beam. This outfit has been placed directly behind the cutting tube A of Fig. 1 in the position of the Petri dish during irradiation and counts have been made at the conclusion of individual experiments. In use, grounded shielding tubes protect the emerging electrode of the collecting plate and the galvanometer leads pass through grounded copper sheathing. The shutter and cutting tube were grounded during both the irradiations and the counting measurements. Standardizations have been carried out (1) with the opening free and (2) with it covered by 0.001 inch aluminum foil and with the chamber evacuated. The fact that the two measurements were substantially identical after allowance had been made for absorption in the foil indicates that the potential developed between the collecting plate and the grounded case was insufficient to interfere seriously with the accuracy of measurement by drawing ions from the air in the chamber. The principal errors in the electron counts probably arise, therefore, from the reflection of electrons by the collecting disk and from inhomogeneity in the cathode ray stream over the irradiated area. No effort has been made to introduce corrections for reflection but shifts of the collecting chamber over the area of irradiation indicated that at the distance of the plate from the mouth of the tube, inhomogeneity of beam was unimportant.

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[3] See also Thaller, R., *Physikal. Zeit.*, 1928, **29**, 841.

Measured galvanometer currents were corrected for the relative areas of the irradiated surface and the opening in the collecting chamber and were expressed in electrostatic units. Taking one E. S. U. as equivalent to 0.21×10^{10} electrons, the numbers of electrons incident per cm.^2 of irradiated surface are recorded in Table I.

The absorption of electrons in the bacteria themselves can scarcely be measured. Because this absorption, like that of X-rays, is not a function of the state of chemical combination it can, however, be estimated from measurements upon cellophane as a substance composed only of light atoms and having approximately the density of bacteria. *It is well known from studies of β-rays that the absorption* of a stream of electrons possessed of different velocities rarely follows any simple law.[4] Measurements of the absorption of the cathode ray beam used in these experiments in successive thicknesses of cellophane lie on a curve intermediate between a straight line and a simple exponential curve. Considering the extrapolation involved in estimating the absorption in a single bacterium from the absorption in a sheet of cellophane (0.0009 inch thick), it has not seemed significant to get more than the order of magnitude of this absorption by a linear interpolation from measureable thicknesses. This procedure has led to the conclusion that there will be an absorption of about 0.008 of the electrons striking either a cylindrical bacterium 0.5μ in diameter and 2μ long or a coccus having a radius of 0.4μ.

DISCUSSION

Whether more than one electron is needed to kill a bacterium can be told from the shape of the survival curves. Expression (2), being a simple exponential, will give a straight line as a graph on semilogarithmic paper. Plotted in this way, the equations for multiple hits yield curves which depart further from a straight line the larger the number of requisite absorptions (Text-fig. 1). Some of the data of Table I are shown graphically in Text-figs. 1 and 2. Within experimental limit the results of the best measurements lie on straight lines. From this fact alone it is to be concluded that the absorption of one electron is sufficient to kill a bacterium. The best obtainable data with *Staphylococcus aureus* depart appreciably from a straight line through the origin. This departure is to be expected, however, for microscopic examination shows that even great dilutions contain many clusters of two or more individuals. The effect of a short incubation of *B. coli* between spreading and irradiation is shown by the top curve of Text-fig. 1. Cell multiplication which has taken place gives a curve resembling but more extreme than that from the coccus.

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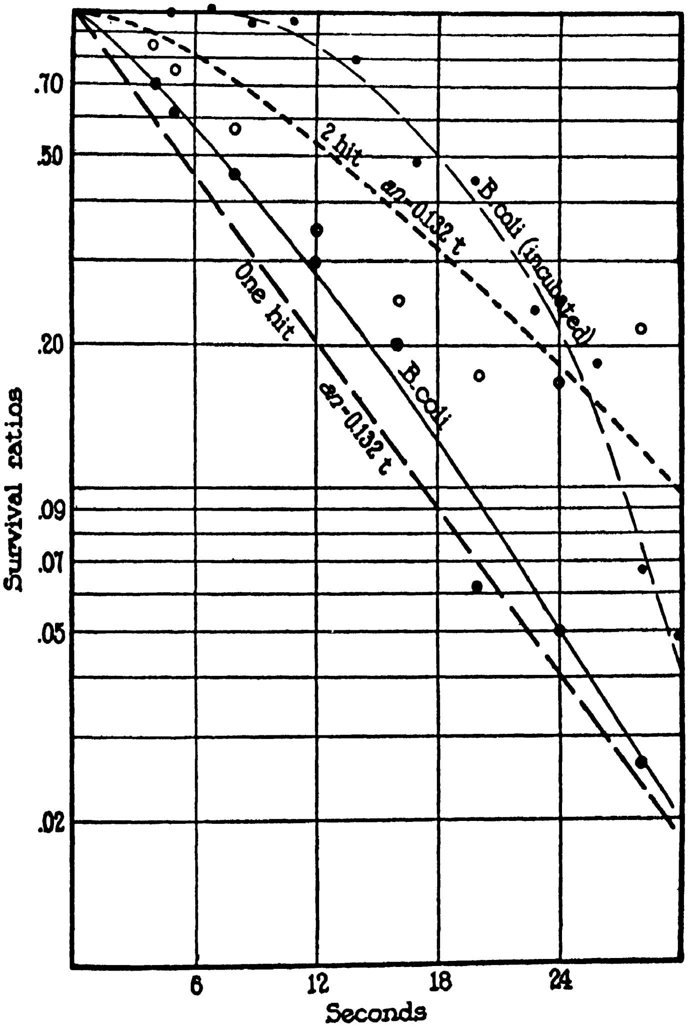
[4] *Cf.* for instance Rutherford, E., Radioactive Substances and their Radiations, London, 1913, Chapter V.

Although one hit kills, it is not necessarily true that every one does so. In Experiment (1) of Table I, 1.17×10^{10} electrons per second



TEXT-FIG. 1. The black circles of this figure are the survival ratios of Experiment (1) of Table I; the open circles refer to Experiment (5) with staphylococci.

are incident upon the irradiated area of 7.07 cm.^{2} The average number of electrons absorbed per second by a cylindrical bacillus $0.5\mu \times 2\ \mu$ is then

$$\frac{1.17 \times 10^{10} \times 1.00 \times 10^{-8}\ \text{cm.}^2 \times 0.008}{7.07\ \text{cm.}^2} = 0.132$$

and Equation (2) as applied to this experiment becomes

$$\frac{A_1}{A_0} = e^{-0.132\,t}$$

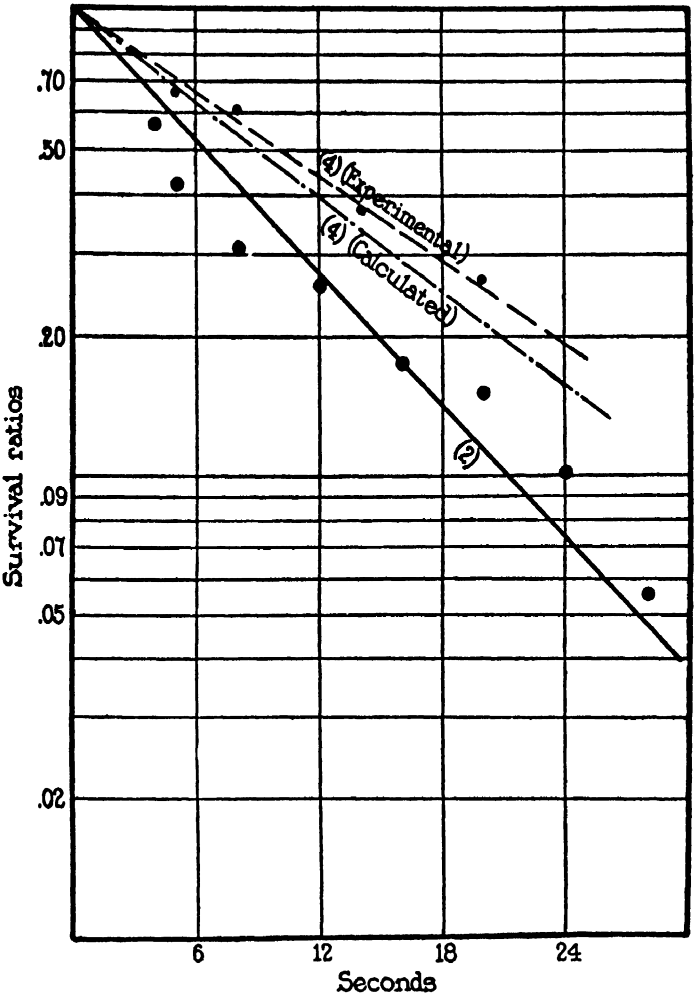
The graph of this expression in Text- fig. 1 lies close to, though somewhat below, the experimental survival ratios. The curve of the corresponding equation if two hits are needed to kill (Equation 3) is shown as the thick dotted line of the same figure. Similar relations prevail in the other standardized experiments. The average number of absorptions per bacterium per second as calculated and observed are listed in Table II. In each instance the theoretical number exceeds the experimental by about 15 per cent. On its face this would mean that only 85 per cent of the bacteria which absorbed electrons were killed, though whenever death was brought about, one electron was sufficient to accomplish it. In view of the inaccuracy of the measurements involved in standardization, however, too much importance cannot be attached to this percentage of killed organisms. It is perhaps safe merely to conclude that the number of bacteria killed is of the same order of magnitude as the number which absorb electrons.

TABLE II

Results from Standardized Experiments

Experiment (Table I)	Electrons absorbed per bacterium per second	
	Observed	Calculated
(1) *B. coli*	ca 0.112	0.132
(3) *B. aert.*	.056	.067
(4) " "	.068	.077
(5) *S. aureus*	ca .105	.114

The similarity in the curves for *B. coli* and *B. aertryke* might be expected from their similarity in size and shape. Though none of the experiments using *Staphylococcus aureus* gave results as uniform as with the two bacilli, the observed greater resistance of this bacterium is adequately accounted for by its smaller size (Table II).



Text-Fig. 2. The data of this figure refer to Experiments (2) and (4) with *B. aertryke.*

CONCLUSIONS

1. For the two motile bacilli, *B. coli* and *B. aertryke,* the absorption of a single 155 K.V. electron is sufficient to cause death. Furthermore,

all, or nearly all, the electrons absorbed are lethal. The same is undoubtedly true of *Staphylococcus aureus.* In addition to providing a quantitative picture of the interaction of bacteria and cathode rays, these results suggest that radiation of the energy content used in our experiments is not suitable for altering the inheritable characteristics of bacteria.

2. The differences in sensitivity to cathode rays shown by the bacteria studied can be explained by the purely physical factor of size.

3. Counts giving significant conclusions concerning killing rates can be obtained only if there is no clumping of the cells when spread and only if the cells are not allowed to multiply before irradiation. Both these precautions seem rarely to have been met in the experiments that have in the past been made with X-rays and other forms of radiation.

EXPLANATION OF PLATE 30

FIG. 1. A photograph showing the anode end of the cathode ray tube and the apparatus used in irradiating the agar plates.

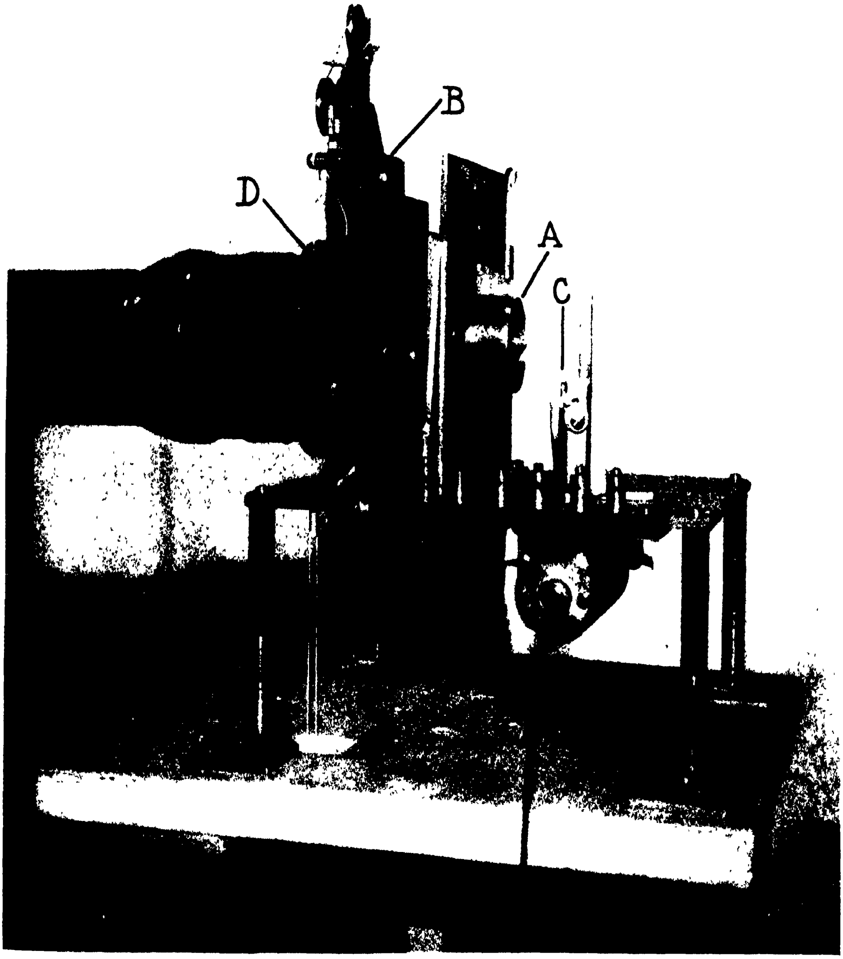


Fig. 1

(Wyckoff and Rivers Effect of cathode rays upon bacteria)

[Reprinted from RADIOLOGY, JOURNAL OF THE RADIOLOGICAL SOCIETY OF NORTH AMERICA, July, 1930, Vol. 15, No. 1, pp. 42-48]

A SIMPLE SELF-RECTIFYING GAS X-RAY TUBE

BY RALPH W. G. WYCKOFF, PH.D., AND J. B. LAGSDIN

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

This tube was originally developed because of the need in some biological experiments for a constant and more intense source than is given by the commercially available X-ray tubes. It has proved to be so simple and reliable in everyday operation, however, that in this laboratory it has replaced commercial tubes for the preparation of the different kinds of photographs used in crystal analysis.

Self-rectifying gas tubes have been made familiar to many through the Shearer tube, as manufactured by the firm of Adam Hilger. There is, however, a wide range of tube dimensions over which self-rectification can be obtained. The amount of high voltage current which a tube will pass unilaterally depends upon its total volume, the size of its cathode, the shape and size of the anode end and other details of its design. A number of forms have been made and tried out in this laboratory. The one to be described is undoubtedly not the best possible, but it has fulfilled our requirements sufficiently well to make further studies unnecessary at this time. The tube has two advantages over the usual type: it is much simpler and cheaper to make; at the same time, it seems from our experience to give steadier operation with a more intense useful beam.

A gas tube was adopted only after long experience with hot cathode metal tubes. Where, as in crystal analysis, purity of spectrum is important and a metal tube is to be in daily use, it has been found easier to build a controllable gas tube than to prevent tungsten sputtering in one of the electron type.

A photograph of the tube is shown in Figure 1. Drawings of the essentials of its construction are reproduced in Figures 2-*A* and 2-*B*. It is composed of three parts, the anode end, A, with target, *a*, the cathode C and the glass tube B which serves to connect the two ends while keeping them insulated from one another.

Except for the water jacket, *j*, and the removable target, *a*, the anode end is machined from a single piece of solid brass rod. Into this is threaded and soldered the pump connection *b*. The cap, *c*, projects into the glass, B, and protects a section of it from sputtering. Flat places, *d*, are machined on either side of the anode block and holes, *e*, cut to provide windows for the emitted X-rays. In tubes for structure analysis these windows have been rectangular, as in Figures 2-*A* and 2-*B*; for some biological irradiations they have been circular with a diameter of 5/16 inch. They have been covered with 0.001 in. aluminum foil which inspection showed to be free from holes. Care

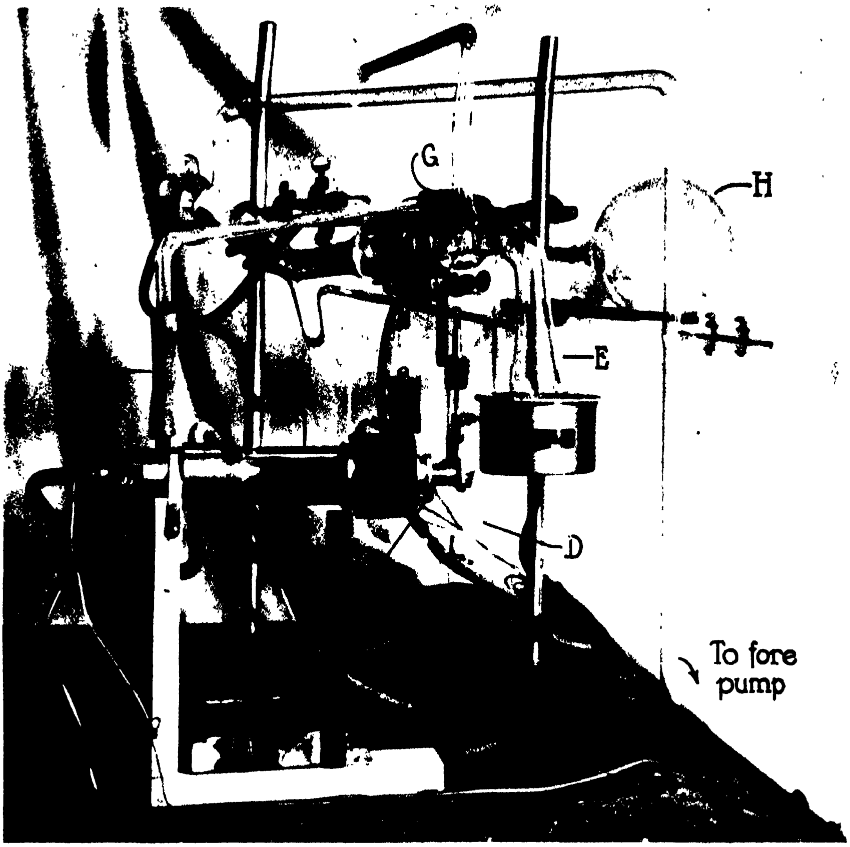


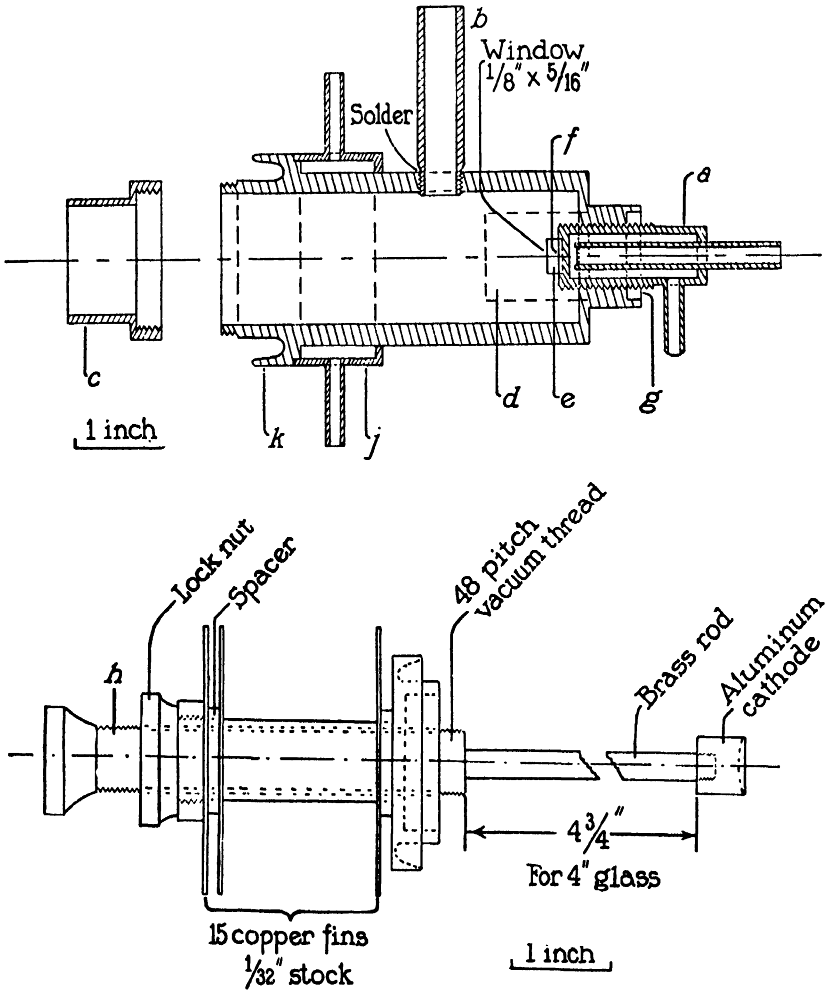
Fig. 1. A photograph of the X-ray tube and its evacuating system. (See Figures 2-*A* and 2-*B*)

must be taken in mounting these windows to be sure that the cementing wax holds the foil smoothly and tightly. If the smallest bits of wax or dirt get inside the tube, it will be erratic in operation until cleaned. Furthermore, if wax gets unevenly under the windows, they will crack and develop leaks as the tube is successively exhausted and filled with air. Hard de Kotinsky cement or white wax has proved best for these seals. Imperfectly made windows, in our experience, account for a majority of the difficulties with these X-ray tubes. The anticathode, *a*, is a hollowed-out, water-cooled brass rod threaded into the anode block. The metal to serve as anticathode is either silver soldered or spun into its face, *f*. It is convenient to have several targets faced with different metals which can be used interchangeably. The seal is effected by filling the cup *g* with a suitable wax. Black piceïn, as a relatively soft wax which wets brass well, is very satisfactory for this joint. In some early tubes the anticathode was carried on a carefully made and greased vacuum thread. Owing to the difficulty of keeping the interior of the tube free from traces of grease, this construction is not to be recommended.

Except for the cathode piece itself, which is of aluminum, and the radiating copper fins, the cathode is of brass. After long use the aluminum end, which should be slightly concave, may become so pitted as to need refinishing. The size of the focal spot will depend upon the distance between target and cathode (ca. 1.75 in.). In order to be able to adjust this focus without destroying the vacuum in the tube, the cathode is mounted on the vacuum threaded screw *h*. When well made and lubricated with a good rubber grease, this thread is completely vacuum-tight. The connecting pyrex glass tube, B, is about four inches long for voltages up to 40,000 volts; with higher voltages a six-inch tube has been used. During operation this glass will become warm. On account of the resulting temperature difference between it and the water-cooled metal end at *k*, a joint made of the best quality red sealing wax is superior to de Kotinsky cement.

In order that apparatus may be brought close to the target, only the cathode is maintained at high potential. Adequate electrical insulation is obtained by making the support *m* of Figure 1 from hard rubber.

For voltages at least as high as 60 K.V. this tube gives perfect self-



FIGS. 2-*A* and 2-*B*. Drawings of the essentials of construction of the X-ray tube. The anode end is shown in 2-*A* (*above*), the cathode in 2-*B* (*below*).

rectification with currents of 10 milliamperes. We have not sought to use higher voltages nor have greater current inputs been tried for. In trial runs currents as high as 20 ma. have been used satisfactorily for short periods but for continuous service at such energy consumption the windows should be water-cooled. In routine work we use a current of ca. 5 ma., with a voltage of ca. 40 K.V. This output is set not by the capacity of the tube but by the fact that it gives sufficiently short exposure times and by the belief that the dangers to the experimenter from the stray radiation with stronger beams are not worth the convenience of still quicker photographs. The beam from a copper target obtained under the foregoing conditions will kill in 20 seconds half of the bacteria it strikes at a distance of two inches from the window and would produce corresponding injury to other living cells in its path. It is obvious that unless care is taken the operator may be seriously injured by these rays. With a good and large crystal such as calcite, a satisfactory 30° oscillation spectral photograph can be made in from one to two minutes; only rarely do the exposures for small crystals or poorly reflecting faces exceed one hour.

The Vacuum System

For the satisfactory operation of a gas tube the evacuating system is as important as the tube itself. Since the output of X-rays depends on the gas pressure, some device must be used to hold this constant. If a steady output is not desired, one of the several sorts of slow gas or air leaks that were developed for early X-ray tubes could be employed. It is as easy and much more satisfactory to make use of a pressure regulator. Several[1] have been suggested. Each is a valve placed between the tube and the vacuum pump and actuated by a solenoid through which flows current from the primary of the high tension transformer. When the gas pressure in the tube rises, more current is consumed. Acting through the solenoid, this increased current opens the valve and allows the pump to exhaust the tube till the pressure has fallen to its previous level. A drawing of the regulator we use is shown in Figure 3. In the photograph (Fig. 1), it is to be seen at D

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[1] R. M. Bozorth, Jour. Am. Chem. Soc., 1927, XLIX, 971; F. E. Haworth, Jour. Opt. Soc. Am., 1929, XIX, 79; T. N. White, Jour. Sci. Inst., 1930, VII, 99.

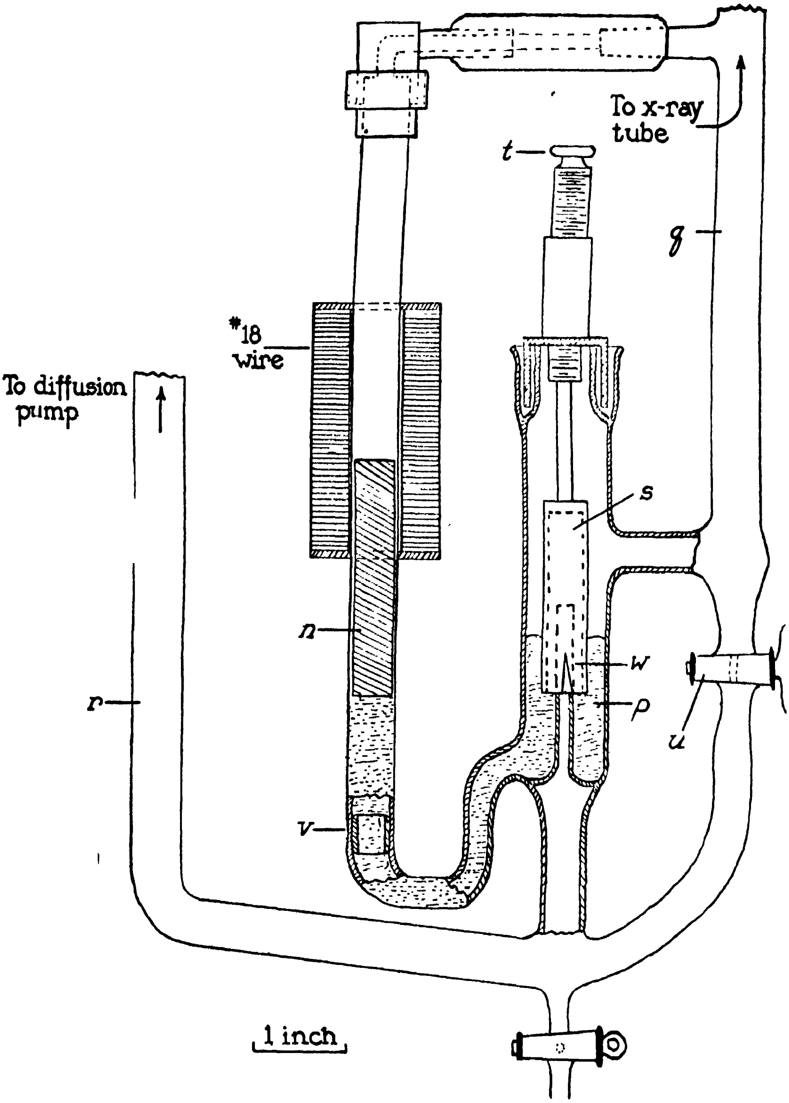

FIG. 3. A drawing showing details of the gas pressure regulator D of Figure 1

between the tube and the mercury pump E. The iron core, *n*, floats on the surface of mercury. When lifted by the solenoid the mercury level in *p* drops and connection is established between *q* and *r* through slots,[2] *w*. The tube current regulated in this way is approximately adjusted by raising and lowering the iron cap, *s*, using the vacuum threaded screw, *t*. Accurate setting is effected by altering the current through the solenoid, which is connected in parallel with a variable resistance in the primary circuit of the transformer. The pull on the core may be varied by changing the height of the solenoid. When

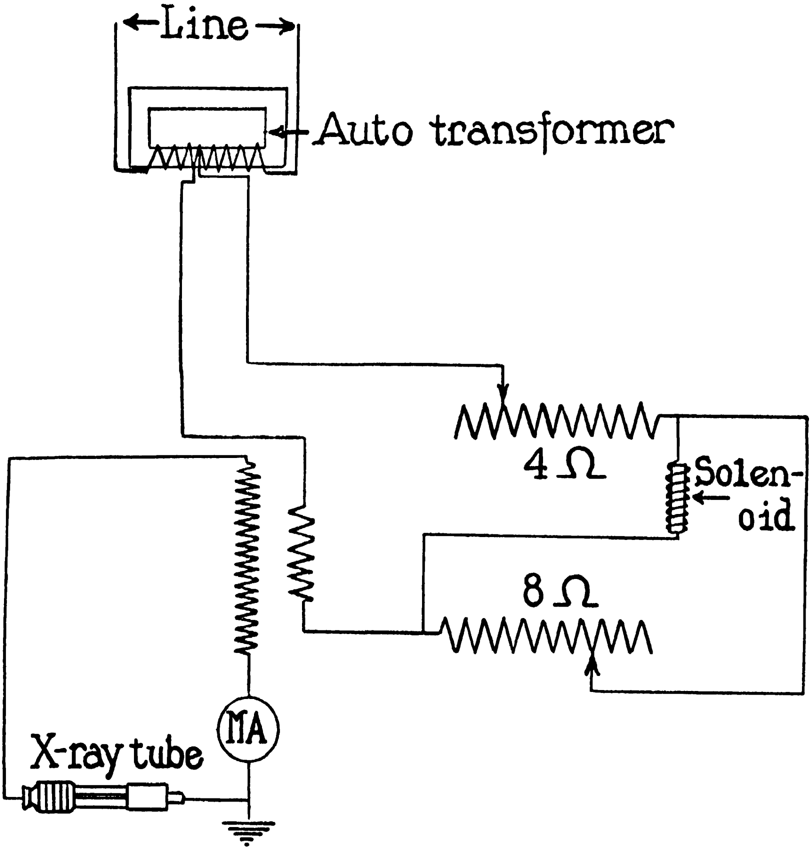

FIG. 4. An electrical circuit suitable for the operation and control of the X-ray tube.

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[2] T. N. White, *op. cit.*

trial has shown the most sensitive position, it can be held in place with a ring of insulating tape wound around the glass tube. The characteristics of a suitable electrical circuit are shown in Figure 4.

The stopcock *u* is left open while the system is pumping to about the desired pressure; during operation it is closed. If small particles of grease or dirt accidentally get into the X-ray tube, the consequent large and erratic evolution of gas may cause the iron core to jump violently. To prevent breakage of the containing arm, a carefully cleaned piece of rubber tubing is shellacked into place at *v* to serve as a bumper.

In breaking in a new tube, it is best to evacuate it till it is completely hard and then to raise the voltage by steps till no current is passed, even momentarily, at the highest potential to be used. For 60 K.V. this should not take more than ten or fifteen minutes if the tube is clean inside and its seals properly made. The voltage should then be reduced to a low value, the stopcock *u* closed and the screw *t* manipulated to allow the tube gradually to soften. As soon as it begins to pass rectified current, as indicated by the milliammeter, the voltage should be raised slowly to the desired value. Frequent use of the stopcock probably will be needed to control the gas pressure until adjustment has been made for the current and voltage to be used. If the tube and system are tight, the whole procedure should not require more than half an hour. We follow the same general routine of complete evacuation and slow raising of voltage every time the tube is to be used. As long, however, as the output is not to be changed, the screw, *t*, need not be adjusted and ordinarily the tube is ready for use within ten minutes of the time the pumps are started.

For steadiest operation it is better not to have the system absolutely tight. Satisfactory results have been obtained by introducing a pair of rubber junctions of nitrometer tubing at F and G of Figure 1. The glass-rubber and metal-rubber surfaces are shellacked but the rubber itself is not painted. The consequent flexibility makes connection of the tube easy and the rubber seems sufficiently porous to give a suitable leakage of air. If the mercury pump is fast, it is perhaps better to increase the volume by introducing a flask such as H.

Where a very steady output is desired, a controllable air leak may be added and a mercury trap cooled in alcohol and solid CO_2 interposed between diffusion pump and tube. With this arrangement a tube will operate day after day for hours at a time with a maximum current fluctuation of ca. 0.1 ma., provided occasional adjustment is made of the solenoid rheostat to take care of gradual hardening after evacuation. When equal voltages are maintained across a tube and equal currents through it, ionization measurements with an air chamber made on successive days have been found constant within the accuracy of the method (ca. 2–3 per cent).

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X-RAY DIFFRACTION DATA FROM SEVERAL MONO-ALKYL SUBSTITUTED AMMONIUM IODIDES

BY RALPH W. G. WYCKOFF

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, January 25, 1930)

Structures have been suggested¹) for the simpler members of this series. The present note contains additional data from some of these compounds and gives measurements on similar crystals having as many as 12 carbon atoms. These data consist of spectral reflections with copper and with molybdenum radiation. A few Laue photographs have also been obtained from single crystals.

With one exception the necessary amines were prepared by R. E. Marker of this Institute. They were synthesized from appropriate alcohols of the best obtainable purity and gave either boiling or melting points in substantial agreement with accepted values.

As in the previous investigation, single crystals developed as scums upon the surface of a desiccated solution. The alkyl ammonium halides dissolve both in water and in ethyl alcohol, though their solubility in water falls off rapidly with increase in length of the carbon chain. Crystalline materials were obtained from alcohol solutions to which a small amount of water had been added. Under favorable conditions of evaporation, the surface of such a solution in a beaker will become covered with a thin but tough membrane which has the X-ray characteristics and all the optical properties of a single uniaxial crystal. Efforts to prepare scums of the iodides containing 14, 16 and 18 carbon atoms in the molecule have not been successful. Though these compounds will take up water to form a gelatinous paste resembling starch, even their most dilute solutions seem to be in reality colloidal. A few individual crystals of minute size have been obtained from solutions of $NH_3(C_{14}H_{29})J$ in absolute alcohol and it is

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1) S. B. Hendricks, Z. Krist. 67, 106. 1928; 68, 189. 1928.

hoped that such specimens will furnish useful data concerning the longer chains.

Series of comparison photographic spectra against (001) of β-Al_2O_3 (d_{001} = 11.24 Å) have been made with copper *K* radiation from butyl, amyl, hexyl, heptyl, octyl, decyl, undecyl and, dodecyl ammonium iodides. Relative intensities of the observed orders from the basal (scum) surfaces and the spacings normal to them are recorded in Table I. Transmission spectral comparisons against (100) of *NaCl* have been made through samples of the butyl, amyl, hexyl and octyl ammonium iodides. All gave a_0 = ca. 5.18 Å. The crystals of these substances are usually either too imperfect or too fragile to yield good Laue photographs or else they exhibit twinning. Excellent though faint photographs have nevertheless been prepared from amyl ammonium iodide. They are very simple and seem to have perfect four-fold symmetry when the X-ray beam is normal to the scum surface. In common with the transmission spectral photographs, the only reflections they possess are ones which would arise from a two-molecule tetragonal cell[1]). The height of this cell increases with the number of carbon atoms in the compound; its cross section is practically constant for all members of the series.

Except for three preparations this height increase is ca. 2.10 Å for each carbon atom added to the chain. The heptyl amine used in this investigation was a commercial preparation and the especially large (001) spacing of the substituted ammonium iodide made from it may mean that it is impure. The large difference in spacing between the decyl and undecyl compound and the small difference between the latter and the dodecyl ammonium iodide suggest that these last two preparations are mixtures. To be certain that compounds of this series can form solid solutions with one another, crystals were grown from mixed solutions of butyl and amyl ammonium iodides. The resulting single crystals gave spacings between those for the pure components (d_{001} = 15.99 Å).

The preparation of undecyl ammonium iodide was dimorphous. The scum which forms on evaporation is isomorphous with the other crystals of this series. When removed from the solution, these single

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1) S. B. Hendricks, op. cit.

crystals will remain substantially unaltered for several days in the cold. More or less rapidly they pass over into an opaque mass which, however, maintains sufficient crystalline orientation to give an excellent series of X-ray reflections from the old scum surface. This spacing, about half of that found from the first modification, is $d = 15.84$ Å. Amongst other series of long chain compounds, those containing an odd number of carbon atoms commonly crystallize differently from the others. The measurements of Table I prove that no such distinction exists among the shorter substituted ammonium iodides, though the dimorphism of undecyl ammonium iodide may well be evidence of such a differentiation amongst the compounds with longer chains.

TABLE I

Substance	(001) Spacing	Intensity of (0 0 *n*) when *n* =											Parameter *u*
		1	2	3	4	5	6	7	8	9	10	11	
$NH_3C_4H_9I$	15.30 Å	s	ab	f+	s	m+	m−	ab	f+	f+	f	—	0.110
$NH_3C_5H_{11}I$	17.42	s	f−	f−	s	m+	m	f	—	—	—	—	.100
$NH_3C_6H_{13}I$	19.50	s	f	ab	s	s	s	m	—	—	—	—	.085
$NH_3C_7H_{15}I$	21.82	m+	m	f	s	s−	s+	s	m	f	—	—	.075
$NH_3C_8H_{17}I$	23.70	m+	m	f+	m	m+	s	s	m+	f	—	—	.070
$NH_3C_{10}H_{21}I$	28.09	—	m	m	ab	f	s	s	s	m	f	—	.060
$NH_3C_{11}H_{23}I$	30.46	—	m	s	ab	ab	s	s	s	s	m	—	.055
$NH_3C_{12}H_{25}I$	31.24	—	s	s	ab	ab	s	s	s	s	m	f	.055

It seems impossible to avoid the conclusion that the iodine atoms in all these compounds have substantially the positions defined by the coordinates $0\ \frac{1}{2}\ u;\ \frac{1}{2}\ 0\ \bar{u}$ of a two-molecule unit. Because of their great weight these atoms in the iodides can be located with considerable accuracy. The relative intensities of the recorded basal reflections lead to values of u which are accurate to within ca. ± 0.004 (Table I).

The attempt to find the positions of the atoms in the substituted ammonium groups brings up a puzzling problem. Considering only this series of crystals, it would be natural to select a two-molecule tetragonal arrangement in which carbon and nitrogen atoms lie on the same vertical axes as the halogen atoms and thus give rise to straight $C_nH_{2n+1}NH_3$ chains. In such a structure the increase in height of the unit cell resulting from the addition of one carbon atom to the chain would be twice the separation of adjacent carbon atoms. This increment in Z is apparently 2.10 Å with a corresponding carbon-to-carbon distance of 1.05 Å. The fact that many cases are known where this atomic separation largely exceeds 1.05 Å is evidence against a straight chain. An important argument against such chains in these compounds is moreover furnished by the structure[1] of the hydrocarbon $C_{29}H_{60}$. In this crystal the contribution to the cell height of a pair of carbon atoms in the same chain is greater than in the substituted ammonium halides. At the same time, examination of the relative intensities of side spectra seems to prove conclusively that the chain cannot be straight. It is unlikely that the carbon chains in the hydrocarbons and the halides are sufficiently different to permit a straight chain in the latter.

At the same time, it is impossible to fit a zigzag chain into a two-molecule tetragonal structure, nor does there seem to be a larger tetragonal cell which can approach the two-molecule unit in the arrangement of its halogen atoms and give zigzag carbon chains. Neither are the spectral and Laue photographs sufficiently complex to admit of the assumption that the tetragonal optical and X-ray properties result from twinning. Further work will be required to tell whether this means that the simple tetragonal form of the structure is fixed by the arrangement of the halogen ions and the NH_3 groups with the carbon atoms having either a lower symmetry or a more or less haphazard distribution about vertical axes[2]. When purer compounds have been obtained, these measurements will be extended in an effort to throw light upon this question.

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1) A. Müller, Pr. Roy. Soc. **120A,** 437. 1928.

2) The problem met here is of the same character as that encountered in placing the ethyl ammonium groups in $(NH_3C_2H_5)_2SnCl_6$ [R. W. G. Wyckoff, Z. Krist. **68,** 231. 1928]. In this hexagonal crystal, either the $NH_3C_2H_5$ groups are straight chains lying on trigonal axes or else the symmetry and simplicity of the X-ray data obtainable from these crystals are much greater than those of the crystals themselves.

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August, 1930, Vol. 15, No. 2, pp. 241–244]

THE CRYSTAL STRUCTURES OF TRIMETHYL AND DIMETHYL ETHYL SULPHONIUM CHLOROSTANNATES AND OF METHYL TRIETHYL PHOSPHONIUM CHLOROSTANNATE

BY ROBERT B. COREY, PH.D., AND RALPH W. G. WYCKOFF, PH.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

Data upon the structures of several substituted ammonium chlorostannates[1] have already been published. The present determinations continue these studies by replacing the substituted ammonium radicals with corresponding sulphonium and phosphonium groups.

All three of the crystals described in this paper appear to be optically isotropic and cubic in form. Although no previous crystallographic description of them could be found, the corresponding chloroplatinates[2] are reported to form optically isotropic octahedra or octahedra with small hexahedral faces. Only octahedral faces were found on any of the crystals, but Laue photographs of the trimethyl and dimethyl ethyl sulphonium chlorostannates taken normal to (111) show plainly the hemihedry characteristic of Ti (T^h) or T. Although these sulphonium salts crystallized readily, producing clear, well-formed octahedra of fair size, single crystals of methyl triethyl phosphonium chlorostannate large enough for Laue or oscillation photographs were not obtained.

The preparation of each chlorostannate was effected by neutralizing the appropriate substituted phosphonium or sulphonium hydroxide with HCl and adding an aqueous solution of H_2SnCl_6. These hydroxides were made by R. E. Marker of this Institute.

The crystal structures were determined in the usual fashion from Laue, oscillation spectral and powder photographs.

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[1] Wyckoff, R. W. G., Am. Jour. Sci., 1928, XVI, 349.
Idem, Ztschr. Kristallographie, 1928, LXVIII, 231.
Wyckoff, R. W. G., and Corey, R. B., Am. Jour. Sci., 1929, XVII, 239.
Idem, Am. Jour. Sci., 1929, XVIII, 138, 437.

[2] See Groth, Paul, Chemische Krystallographie, Leipzig, 1906, I, 479, 482.

The Crystal Structure of Trimethyl Sulphonium Chlorostannate

The data for this study were obtained from Laue photographs and from oscillation photographs using both copper and molybdenum radiation.

Six comparison photographs were made between the (111) face of $[(CH_3)_3S]_2SnCl_6$ and the (001) face[3] of $\beta-Al_2O_3$ (d_{001} = 11.24 Å.) using copper radiation. The average of eighteen reflections taken from the first, second, and third orders on these photographs gives d_{111} = 7.17 Å., which leads to the length of a cube edge a_o = 12.41 Å. The density as obtained by the ordinary suspension method is ρ_{20° = 1.69. Calculation from this density shows that a unit cube having the foregoing a_o will contain four molecules (m = 4.03). Data from an oscillation spectral photograph using molybdenum radiation are reproduced in Table I. Typical results on an octahedral Laue photograph are listed in Table II. Although the strongest first order reflections have only odd indices, such reflections occur from all sorts of planes so that the fundamental lattice is Γ_c. Of the possible first order spots with one index zero those are found which are (0, even, odd), whereas those that are (0, odd, even) and (0, odd, odd) are invariably absent. This is the distinguishing criterion of Ti-6 (T_h^6),

TABLE I

Typical Reflection Spectral Data upon Photographs of Trimethyl Sulphonium Chlorostannate

[(111) as Principal Spectrum]

Indices	Intensity	Indices	Intensity
111	v.s	220	s
222	f	440	m
333	ff	002	m
444	f	004	v.s
555	ff	113	m
553	ff	224	f+
551	f−	115	s
		226	f

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[3] Pauling, Linus, and Björkeson, Albert, Proc. Nat. Acad. Sci., 1925, XI, 445.

which, therefore, is in all probability the correct space group. The atomic arrangement then is as follows:[4]

Tin atoms: 4b.

Sulphur atoms: uuu and other seven positions of 8h.

Chlorine atoms: xyz and other general positions of Ti-6.

Carbon atoms: x′y′z′ and another set of general positions of Ti-6.

The structure of $[(CH_3)_3S]_2SnCl_6$ is, therefore, similar to that[5] of $[(CH_3)_3NH]_2SnCl_6$. Lack of the necessary F-curve data makes unprofitable an attempt to establish exact atomic positions at this time.

TABLE II

Typical Laue Data from An Octahedral Photograph of Trimethyl Sulphonium Chlorostannate

Indices	Spacing	Wave Length	Intensity
$0\bar{4}5$	2.24 Å.	0.483 Å.	f−
$1\bar{4}4$	2.17	.482	f−
$\bar{3}5\bar{1}$	2.11	.446	v.s
$\bar{5}42$	1.86	.401	f
$34\bar{5}$	1.76	.487	ff
$\bar{5}15$	1.75	.392	m
$25\bar{5}$	1.70	.458	f−
$\bar{3}7\bar{2}$	1.58	.475	f

The Crystal Structure of Dimethyl Ethyl Sulphonium Chlorostannate

Data were obtained from Laue photographs and from oscillation photographs using molybdenum radiation. Six comparison spectra were made between the (111) face of $[(CH_3)_2(C_2H_5)S]_2SnCl_6$ and the (100) face of calcite. An average of sixteen reflections taken from the second, third, and fourth orders gives $d_{111} = 7.39$. From this the length of the unit cube edge is $a_o = 12.80_5$. The density $\rho_{20°} = 1.63$ leads to a unit cube which contains four molecules (m = 4.04). Typical data from an octahedral Laue photograph are contained in Table III. Reflections from an oscillation spectrum are listed in

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[4] Wyckoff, R. W. G., The Analytical Expression of the Results of the Theory of Space Groups, Washington, 1930, 2d Ed., p. 127.

[5] Wyckoff, R. W. G., and Corey, R. B., Am. Jour. Sci., 1929, XVIII, 437.

Table IV. All agree with a four molecule unit. There is a striking similarity between both the Laue and spectral data and those obtained from $[(CH_3)_3S]_2SnCl_6$. Possible first order reflections with one index zero include only those which are (0, even, odd), so that the space group would appear to be Ti-6.

TABLE III

Typical Laue Data from An Octahedral Photograph of Dimethyl Ethyl Sulphonium Chlorostannate

Indices	Spacing	Wave Length	Intensity
$\bar{3}\bar{1}5$	2.16 Å.	0.497 Å.	v.s
$3\bar{5}3$	1.95	.434	m+
$2\bar{5}4$	1.91	.426	ff
$34\bar{5}$	1.81	.488	ff
$1\bar{5}5$	1.79	.384	m
$52\bar{5}$	1.74	.482	ff
$61\bar{5}$	1.63	.426	f
$7\bar{3}\bar{2}$	1.63	.472	ff

TABLE IV

Typical Reflection Spectral Data from A Photograph of Dimethyl Ethyl Sulphonium Chlorostannate

[(111) as Principal Spectrum]

Indices	Intensity	Indices	Intensity
220	s	223	ff
440	m	224	m
111	v.s	225	ff
222	f	226	f−
333	ff	227	ff
444	f−	335	ff
113	m+	336	f
114	ff	445	ff
115	m		

In several of the other substituted chlorostannates which have been investigated, difficulties have been encountered in placing the hydrocarbon groups in positions which were chemically reasonable and which at the same time conformed to the apparent symmetry. In $[(CH_3)_2(C_2H_5)S]_2SnCl_6$ this seems impossible. No cubic group which could explain the observed diffraction effects has the requisite atomic positions. Ordinarily this would indicate that the crystal was only pseudo-cubic. In the present instance, however, no departure from cubic symmetry could be detected by either goniometric or optical observations. Whether this means that the grouping of the atoms within the substituted sulphonium ion does not partake of the total crystal symmetry or whether these groups in their efforts to attain symmetrical arrangements give rise to a large unit containing 32 molecules cannot be told from the existing data.

TABLE V

Data from Powder Photographs of Methyl Triethyl Phosphonium Chlorostannate

Spacing	Intensity	$\sin^2\theta$	Ratio of $\sin^2\theta$	Assigned $h^2 + k^2 + l^2$	Indices	
1.620 Å.	f	0.04802	73.83	..	....	....
1.850	m−	.03684	56.65	56	123 (2)	13.968 Å.
1.950	m−	.03316	50.99	51	155 (1), 117 (1)	13.925
2.209	m	.02583	39.72	40	130 (2)*	13.972
2.338	m	.02306	35.46	35 and 36	135 (1) and 100 (6), 122 (2)	....
2.474	ff	.02058	31.64	32	110 (4)	13.995
2.679	s	.01756	27.00	27	115 (1), 111 (3)	13.920
3.116	ff	.01298	19.96	19 and 20	133 (1) and 120 (2)	....
3.480	f	.01041	16.01	16	100 (4)	13.920
4.233	s	.00704	10.83	11	113 (1)	14.041
						13.963 Å.

* A line having a slightly smaller spacing than 130 (2) was plainly visible on all photographs but was too faint to be measured. Estimation would place it at $h^2 + k^2 + l^2 = 43$ and thus give it indices 335.

The Crystal Structure of Methyl Triethyl Phosphonium Chlorostannate

No success has attended attempts to prepare large crystals of $[(CH_3)(C_2H_5)_3P]_2SnCl_6$. When solutions of H_2SnCl_6 and the substituted phosphonium hydrochloride were mixed the salt appeared as a white, crystalline precipitate consisting of tiny, clear, optically isotropic octahedra. Even when crystals were formed by slow evaporation of very dilute solutions none was produced of sufficient size to yield Laue or oscillation spectral photographs. For this reason, the data are confined to those given by powder photographs alone. Such data are recorded in Table V. The spacings are the average of those obtained from the measurement of six comparison films against NaCl (Table VI). Pyknometric determination of the density of $[(CH_3)(C_2H_5)_3P]_2SnCl_6$ using benzene as the confining liquid gave $\rho_{20^\circ} = 1.48$. A unit cube having this density and $a_0 = 13.93$ Å. will contain four molecules (m = 4.05).

TABLE VI

Values of a_0 for Methyl Triethyl Phosphonium Chlorostannate as Averaged from Comparison Films with NaCl

Film No.	a_0
1	13.921 Å.
2	13.938
3	13.927
4	13.898
5	13.973
6	13.931
	13.93_1 Å.

No odd order powder lines were found which had other than all odd indices, with the possible exception of the outermost one measured. Other planes might be expected from an arrangement based on the simple lattice Γ_c. It will be remembered, however, that the corresponding ammonium compound, $[N(CH_3)(C_2H_5)_3]_2SnCl_6$, which presumably has the space group T-4, gave data showing no departure from a true face-centered structure of holohedral symmetry. It seems most probable that the structure of $[P(CH_3)(C_2H_5)_3]_2SnCl_6$ is analogous, with tin and chlorine atoms in substantially the same higher symmetry positions that they occupy in $(NH_4)_2SnCl_6$ but with the aliphatic carbon atoms in 4f and the general positions of T-4.

CONCLUSION

Crystals of trimethyl sulphonium, dimethyl ethyl sulphonium and of methyl triethyl phosphonium chlorostannates are cubic with $(NH_4)_2SnCl_6$, or CaF_2, arrangements of their ions. The trimethyl sulphonium and the methyl triethyl phosphonium chlorostannates are like the corresponding substituted ammonium compounds, but with somewhat larger unit cells. Though a departure from cubic symmetry could not be observed for the dimethyl ethyl sulphonium chlorostannate, no set of positions for all its atoms compatible with this high symmetry and its apparent simple structure could be found.

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CONFIGURATIONAL RELATIONSHIPS OF PHENYLATED CARBINOLS

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(Received for publication, April 8, 1930)

All the carbinols which have been directly correlated to lactic acid have been strictly aliphatic. A number of carbinols with phenyl groups has been studied but none correlated. Hewitt and Kenyon[1] pointed out that the phenylated carbinols possess much higher activities than the corresponding aliphatic ones of the same number of carbon atoms, and that the enhancement apparently increases as the phenyl group approaches the asymmetric carbon atom. But they made no direct correlations and must have assumed that the phenyl group does not cause an inversion of the sign of rotation.

This paper deals with the correlation of a number of carbinols containing a phenyl group at different distances from the asymmetric carbon atom. The first studied was ethyl-β-phenethyl carbinol II which was correlated to lactic acid in the manner shown in the accompanying diagram.

The results of these correlations show that levo-ethyl-β-phenethyl carbinol II and levo-ethyl-β-cyclohexethyl carbinol III are related to levo-ethylpropyl carbinol, that a phenyl group in the β position to the asymmetric carbon atom causes no inversion of the sign of rotation, but rather an enhancement, and that a cyclohexyl group likewise in the β position is roughly equivalent to a normal hexyl group. Further, the presence of a double bond in the α, β position as in I causes an inversion of sign just as in all other similarly unsaturated carbinols.[2]

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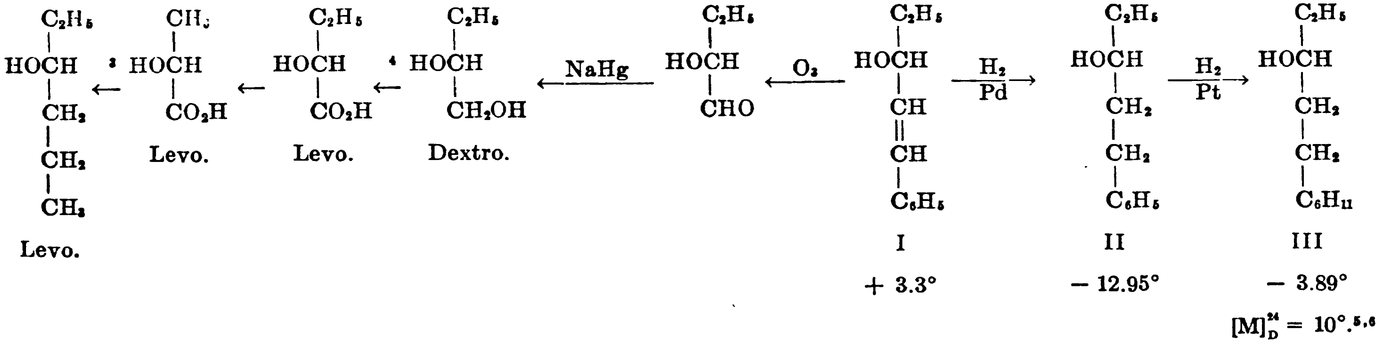
[1] Hewitt, L. F., and Kenyon, J., *J. Chem. Soc.*, **127**, 1094 (1925).

[2] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **83**, 579 (1929).



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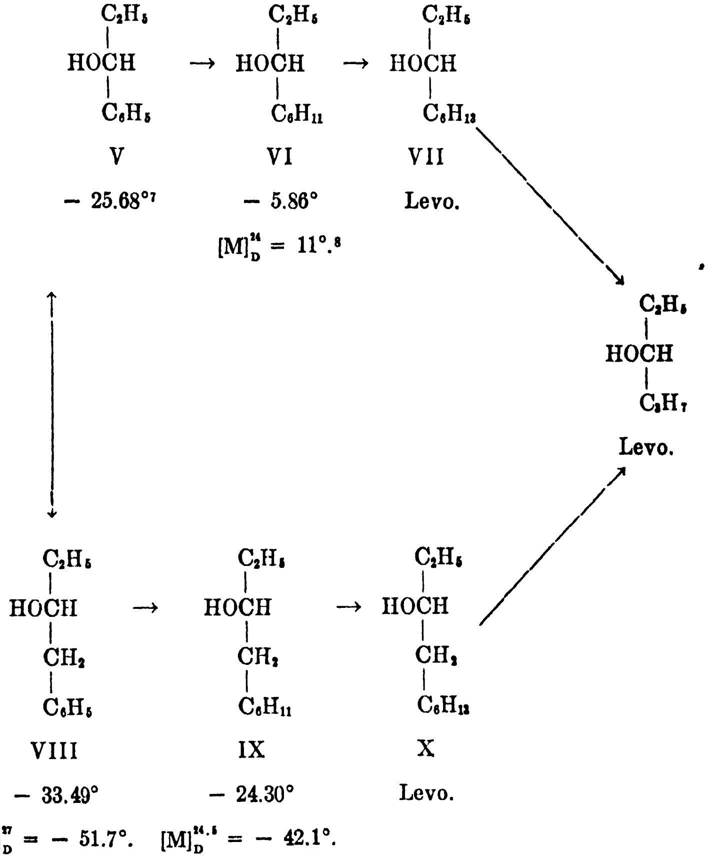
[3] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **76**, 415 (1928).

[4] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **74**, 343 (1927).

[5] Calculated approximately from the maximum value of Hewitt and Kenyon's ethyl-β-phenethyl carbinol.

[6] Hewitt and Kenyon found $[M]_D^{20}$ for ethyloctyl carbinol to be 10.7.

The fact that a cyclohexyl group is about equivalent to a normal hexyl group made it possible to correlate carbinols with the phenyl group directly attached to and once removed from the asymmetric carbon atom with a reasonable degree of probability. The reduction of the phenyl group in each case resulted in a decrease in activity but no change of sign.



The correlations VI to VII and IX to X rest upon two considerations: First, the fact that direct correlation showed that a cyclohexyl group in the β position is about equivalent to the normal hexyl group, no inversion of sign taking place, and second, that the cyclohexyl group is a saturated radical in its chemical nature and resembles a straight chained group rather than a branched chain or a polar radical (with the possible exception of the $C_6H_{11}CH_2$ group). Thus it merely becomes the heavier group, and by the rule of Levene and Haller[9] should be related to other carbinols having the heavier group in the same position. But these correlations must be considered tentative until the relation between cyclohexyl and normal hexyl groups has been established by direct evidence.

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[7] This material was prepared by Levene and Mikeska (Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, 70, 355 (1926)).

[8] Calculated approximately from Pickard and Kenyon's data (Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, 99, 45 (1911)). Pickard and Kenyon give $[M]_D^{20} = 10.6$ for ethylhexyl carbinol.

The effect on the rotation of the distance of the phenyl group from the asymmetric carbon atom as yet cannot be summed up in the form of one simple rule. Hewitt and Kenyon report the following molecular rotations for the methyl series.

A	
$CH_3CH(OH)Ph$	50.9
$CH_3CH(OH)CH_2Ph$	36.1
$CH_3CH(OH)CH_2CH_2Ph$	21.3

B		Δ (A − B)	Ratio $\frac{A}{B}$
$CH_3CH(OH)C_6H_{13(n)}$	12.7	38.2	4:1
$CH_3CH(OH)CH_2C_6H_{13(n)}$	12.9	23.2	3:1
$CH_3CH(OH)CH_2CH_2C_6H_{13(n)}$	13.7	7.6	1.5:1

Thus the authors conclude that the enhancing effect of the phenyl group drops with the increase in the distance.[10]

The state of affairs seems to be different in the ethyl series.

A		B	
$C_2H_5CH(OH)Ph$	35.2[1]	$C_2H_5CH(OH)C_6H_{13(n)}$	10.6[1]
$C_2H_5CH(OH)CH_2Ph$	51.7	$C_2H_5CH(OH)CH_2C_6H_{13(n)}$	10.6[11]
$C_2H_5CH(OH)CH_2CH_2Ph$	31.0[1]	$C_2H_5CH(OH)CH_2CH_2C_6H_{13(n)}$	10.7[1]

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[9] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **79**, 475 (1928).

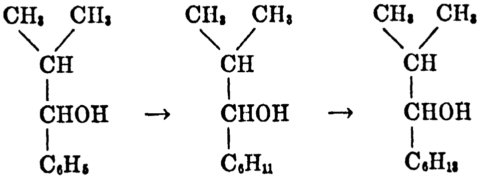
[10] Compare Kuhn, W., *Ber. chem. Ges.*, **63**, 199 (1930).

[11] Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, **103**, 1933 (1913).

C		Δ (A−B)	Δ (A−C)	$\frac{A}{B}$	$\frac{A}{C}$
$C_2H_5CH(OH)C_6H_{11}$	11[8]	24.6	24.2	$\frac{3.3}{1}$	$\frac{3.2}{1}$
$C_2H_5CH(OH)CH_2C_6H_{11}$	42.1[12]	41.1	9.6	$\frac{4.9}{1}$	$\frac{1.25}{1}$
$C_2H_5CH(OH)CH_2CH_2C_6H_{11}$	10[6]	20.3	21	$\frac{2.9}{1}$	$\frac{3.1}{1}$

Hence the regularity observed in the methyl series seems to be lacking in the ethyl series. The difference may be real, but there is an equal probability that it is only apparent due to the fact that the values accepted by Hewitt and Kenyon as the maxima, are in reality the values for the substances only partially resolved.

In all the above cases, only one polar group is present; the phenyl group. A study of isopropylphenyl carbinol was of interest therefore because of the two different polar groups. Reduction here caused an inversion of sign as well as a decrease in activity.



XI — XII — XIII

− 9.01°[7,13] — + 2.75° — Dextro.

Due to certain irregularities in the isopropyl series, the correlation of XII to XIII is tentative. Also, it is not possible as yet to cor-

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[12] The irregularity of rotatory power here shown by these cyclohexyl carbinols may be related to the irregularity of dissociation constants exhibited by a similar set of acids.

	K at 25°*
$C_6H_{11}CO_2H$	1.34×10^{-5}
$C_6H_{11}CH_2CO_2H$	2.36×10^{-5}
$C_6H_{11}CH_2CH_2CO_2H$	1.34×10^{-5}

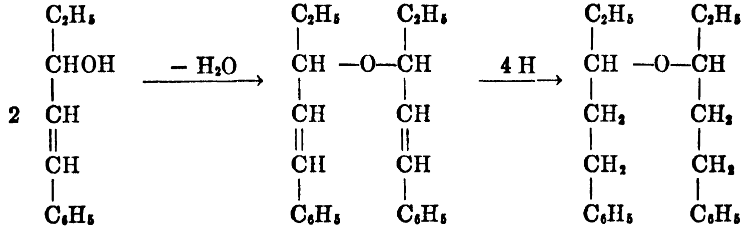
* Zelinsky, N., and Izgaryschew, N., *Chem. Zentr.*, 1, 532 (1909).

[13] Highest value found α_D^{22} = +20.23°.

relate XIII with the normal secondary carbinols. A further investigation in this direction is now in progress.

During the reductions of these phenylated carbinols, considerable amounts of hydrocarbons were formed. Adams' platinum oxide catalyst was used with hydrogen at 30 pounds pressure in glacial acetic acid solution. It was found that in those cases where the phenyl group was directly attached to the asymmetric carbon atom, as much as two-thirds of the material was reduced to the corresponding hydrocarbon. Where the phenyl group was once removed, only about 8 per cent was reduced to the hydrocarbon and where twice removed, practically none.

Difficulty was experienced in isolating active ethylstyryl carbinol in a pure state, because the carbinol readily forms a dimolecular ether which possesses a high activity as compared with that of the carbinol. The unsaturated character of the ether was apparent from its absorption of bromine and hydrogen. The reduction was carried out with Adams' platinum oxide catalyst, colloidal palladium being without effect. The reduced ether displayed a greatly decreased activity.



$$2\ C_2H_5-CHOH-CH=CH-C_6H_5 \xrightarrow{-H_2O} (C_6H_5-CH=CH-CH(C_2H_5))_2O \xrightarrow{4H} (C_6H_5-CH_2-CH_2-CH(C_2H_5))_2O$$

Dextro in alcohol. Levo in alcohol. Levo in ether ?

Levo in ether. Levo in ether.

EXPERIMENTAL

Ethylstyryl Carbinol

The carbinol was prepared from cinnamic aldehyde by the action of ethyl magnesium bromide.[14] It was converted into the acid phthalate in the usual way with pyridine and phthalic anhydride. This, after two recrystallizations from ether and ligroin, melted at 108–109°, resolidified, and remelted at 160° ± with decomposition.

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[14] We are indebted to Mr. R. E. Marker for the preparation of this carbinol.

Resolution of the Acid Phthalate.—The alkaloid used was cinchonidine, for neither brucine nor strychnine gave crystalline salts. From acetone the salt of one modification crystallized out splendidly. After several recrystallizations from 95 per cent alcohol, the salt was decomposed, and the crystalline phthalate melting at 83.5–85° was examined. In absolute alcohol it had the following activity.

$$[\alpha]_D^{23} = \frac{-0.52° \times 100}{1 \times 17.40} = -3.0°.$$

The activity of various samples ranged between this value and −2.3°. A sample which in absolute alcohol was −2.4° at 27° had the following rotation in absolute ether.

$$[\alpha]_D^{27} = \frac{-0.18° \times 100}{1 \times 16.5} = -1.1°.$$

As the more soluble cinchonidine salt did not crystallize, it was converted to the crystalline phthalate, which had the following rotation in absolute alcohol.

$$[\alpha]_D^{23} = \frac{+0.40° \times 100}{1 \times 16.80} = +2.4°.$$

The usual procedure was employed for the saponification of the phthalate. The carbinol, however, boiled so high that it was extracted with ether rather than steam-distilled. Purification of the carbinol by distillation proved difficult. It did not boil at all constantly. Moreover, there was a large viscous residue,—probably the dimolecular ether, as it turned out to be highly optically active. From the above dextro-phthalate, the following fractions at 1.0 mm. were obtained.

1st fraction, boiling point 97–108°, No. 35.
2nd " " " 108–111°, " 36.

Both were dextrorotatory in absolute alcohol.

$$\text{No. 35.}\quad [\alpha]_{D}^{24} = \frac{+\,0.25^\circ \times 100}{1 \times 19.36} = +\,1.3^\circ.$$

$$\text{No. 36.}\quad [\alpha]_{D}^{24} = \frac{+\,0.25^\circ \times 100}{1 \times 17.92} = +\,1.4^\circ.$$

Redistillation of No. 36 yielded the following fractions at 0.8 mm.

1st fraction, boiling point up to 101°, No. 37.
2nd " " " 101–105°, " 38.
3rd " " " 105–115°, small amount only.
4th " Residue. No. 40.

Both No. 37 and No. 38 were levorotatory in absolute ether.

$$\text{No. 38.}\quad [\alpha]_{D}^{25} = \frac{-\,0.93^\circ \times 100}{1 \times 16.54} = -\,5.6^\circ.$$

No. 38 was redistilled. The fraction which boiled at 0.6 mm. at 112–115° gave the following analysis.

4.505 mg. substance: 13.480 mg. CO_2 and 3.485 mg. H_2O.
$C_{11}H_{14}O$. Calculated. C 81.5, H 8.6.
Found. " 81.2, " 8.7.

In absolute ether it had the following rotation.

$$[\alpha]_{D}^{26} = \frac{-\,0.88^\circ \times 100}{1 \times 16.26} = -\,5.4^\circ.$$

The residue, No. 40, probably the ether, gave the following analysis.

3.210 mg. substance: 10.135 mg. CO_2 and 2.405 mg. H_2O.
$C_{22}H_{26}O$. Calculated. C 86.2, H 8.5.
Found. " 86.1, " 8.4.

In absolute ether it had a high rotation.

$$[\alpha]_{D}^{25} = \frac{-\,11.76^\circ \times 100}{1 \times 16.56} = -\,71.0^\circ.$$

In absolute alcohol it was also levorotatory.

The formation of the ether takes place on distilling and also on standing at room temperature. For this reason it was difficult to be certain of the amount of rotation of the carbinol. Thus No. 35 which was dextrorotatory in absolute alcohol, after several weeks, became strongly levorotatory.

$$[\alpha]_D^{22.5} = \frac{-5.40° \times 100}{1 \times 23.26} = -23.2°.$$

In absolute ether it was also much more strongly levorotatory than the pure carbinol.

$$[\alpha]_D^{24} = \frac{-4.00° \times 100}{1 \times 20.66} = -19.3°.$$

The same carbinol prepared from another lot of dextro-phthalate ($\alpha_D^{24} = +2.3°$) was very carefully fractionated. After several distillations, a fraction was obtained which boiled at 115–118°, mostly at 118° at 0.2 mm. (metal bath at 140–145°). This was colorless, practically odorless, and had the following composition.

3.740 mg. substance: 11.290 mg. CO_2 and 2.905 mg. H_2O.
$C_{11}H_{14}O$. Calculated. C 81.5, H 8.6.
Found. " 82.3, " 8.7.

In absolute alcohol it was dextrorotatory.

$$[\alpha]_D^{23} = \frac{+0.39° \times 100}{1 \times 18.52} = +2.1°.$$

In absolute ether it was levorotatory.

$$[\alpha]_D^{23} = \frac{-1.07° \times 100}{1 \times 15.48} = -6.9°.$$

Without solvent, 1 dm. tube, $\alpha_D^{23} = -3.30°$.

The levo-phthalate ($[\alpha]_D^{24} = -2.4°$) in the same way gave a carbinol that was dextrorotatory. The crude carbinol after drying but before distillation had the following composition.

3.835 mg. substance: 11.575 mg. CO_2 and 2.870 mg. H_2O.
$C_{11}H_{14}O$. Calculated. C 81.5, H 8.6.
Found. " 82.3, " 8.4.

In absolute ether it had the following rotation.

$$[\alpha]_D^{22} = \frac{+\ 1.82° \times 100}{1 \times 17.52} = +\ 10.4°. \quad \text{No. 48.}$$

Doubtless this activity here is too high for the pure carbinol due to contamination by the active ether.

Phenylurethane of Dextro-Ethylstyryl Carbinol.—To 2 gm. of dextro-carbinol No. 48 were added 1.5 gm. of phenylisocyanate. The mixture was gently warmed for 15 minutes on the steam bath. On cooling it set to a crystal cake, which was ground up and washed well with ligroin. After two recrystallizations from absolute alcohol, the colorless needles melted at 112.5–113° and analyzed as follows:

9.600 mg. substance: 0.426 cc. N_2 at 25° ana 753 mm.
$C_{18}H_{19}O_2N$. Calculated. N 5.0 Dumas.
Found. " 5.1.

The urethane had the following rotations.

$$\text{In absolute alcohol } [\alpha]_D^{26.5} = \frac{+\ 2.62° \times 100}{1 \times 2.24} = +\ 117.0°.$$

$$\text{In chloroform } [\alpha]_D^{25} = \frac{+\ 17.47° \times 100}{1 \times 15.04} = +\ 116.1°.$$

Dextro-Butylene Glycol

Ozonization of Dextro-Ethylstyryl Carbinol.—11.4 gm. of carbinol No. 48, dissolved in 30 cc. of pure glacial acetic acid, were ozonized at 0° until the solution no longer added bromine. The decomposition of the ozonide was carried out according to Helferich[15] in the presence of zinc. The zinc was filtered off and carefully washed with ether and glacial acetic acid. The filtrate was concentrated *in vacuo*, neutralized with sodium hydroxide; the zinc hydroxide was filtered off and washed well with alcohol and water. This filtrate, consisting of about 350 cc. of 50 per cent alcohol, was treated with sodium amalgam until the solution no longer reduced Fehling's solution. The mixture was then filtered, neutralized with 10 per cent sulfuric acid and concentrated under reduced pressure. Upon addition of acetone, all the sodium acetate was precipitated. Reconcentration and further addition of acetone produced no further precipitate. The solution was then reconcentrated, water added, and filtered. Again the filtrate was evaporated under reduced pressure. The residual oil was taken up in ether, dried over sodium sulfate, and the ether evaporated. The remaining liquid, after drying at 70° at 0.6 mm. weighed 2.8 gm., a yield of about 25 per cent.

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[15] Helferich, B., *Ber. chem. Ges.*, **52**, 1123 (1919).

Dextro-Butylene Glycol Di-(Phenylurethane).—The above oil was treated with an excess of phenylisocyanate. The mixture warmed energetically by itself and was further heated for 2 to 3 hours on the steam bath. On chilling, only a paste was obtained. After successive extractions with ligroin and later with mixtures of ligroin and ether, most of the benzyl alcohol urethane was removed, and the paste then solidified. After recrystallizing twice from benzene and ligroin, and thrice from dilute alcohol, the fine needles melted at 124–125°.

The mixed melting point with a sample of dextro-butylene glycol di-(phenylurethane) (melting point 124–125°, $[\alpha]_D^{20} = +23.7°$)[16] was 124°–125°. The di-urethane had the following rotation in absolute alcohol.

$$[\alpha]_D^{21.5} = \frac{+1.04° \times 100}{1 \times 4.46} = +23.3°.$$

It analyzed as follows:

4.250 mg. substance: 10.450 mg. CO_2 and 2.395 mg. H_2O.
9.350 “ “ : 0.7644 “ N_2 (Van Slyke).
$C_{18}H_{20}O_4N_2$. Calculated. C 65.9, H 6.1, N 8.5.
Found. “ 66.6, “ 6.3, “ 8.2.

Levo-Ethyl-β-Phenethyl Carbinol

Reduction of Dextro-Ethylstyryl Carbinol.—10 gm. of dextro carbinol No. 48, dissolved in ether, were shaken with aqueous colloidal palladium and hydrogen. After about 6 hours, when the absorption seemed complete, the ether was separated, dried over sodium sulfate, and evaporated. The residual oil was distilled. The fraction distilling slowly at 87–88° at 0.4 mm., solidified in the receiver to long white spears melting at 38–41°, and analyzed as follows:

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[16] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **74**, 349 (1927).

4.395 mg. substance: 13.030 mg. CO_2 and 3.830 mg. H_2O.
$C_{11}H_{16}O$. Calculated. C 80.5, H 9.8.
Found. " 80.8, " 9.8.

In absolute ether it had the following rotation.

$$[\alpha]^{24}_{D} = \frac{-3.14° \times 100}{1 \times 16.72} = -18.8°.$$

Without solvent, 1 dm. tube, $\alpha^{25.5}_{D} = -12.63°$.

Another sample, from a slightly more active phthalate ($[\alpha]^{23}_{D} = -2.9°$) was carefully fractionated. The fraction boiling at 103–107° (rapidly distilled) at 0.1 mm.[17] solidifying as before in the receiver, was considered pure and had the following rotations.

Without solvent, 1 dm. tube, $\alpha^{25}_{D} = -12.95°$, $[\alpha]^{25}_{D} = -13.3°$, $[M]^{25}_{D} = -21.8°$.[18]

$$\text{In absolute alcohol } [\alpha]^{25}_{D} = \frac{-2.63° \times 100}{1 \times 14.74} = -17.8°.$$

$$\text{In absolute ether } [\alpha]^{25.5}_{D} = \frac{-2.93° \times 100}{1 \times 16.48} = -17.7°.$$

α-Naphthylurethane of Levo-Ethyl-β-Phenethyl Carbinol.—To 1 gm. of the saturated carbinol ($[\alpha]^{24}_{D} = -18.0°$ in ether) were added 1.5 gm. of α-naphthyl isocyanate. The mixture crystallized after 1 hour on the steam bath. After chilling, the crystals were washed well with ligroin and thrice recrystallized from aqueous alcohol. The snow-white needles then melted at 82–83° and had the following rotation in absolute alcohol.

$$[\alpha]^{24.5}_{D} = \frac{-1.33° \times 100}{1 \times 14.96} = -8.9°.$$

The analysis (Dumas) gave too high a nitrogen content. The material was therefore recrystallized repeatedly from both chloroform and ligroin, and aqueous alcohol. It then melted at 82.5–83° and analyzed as follows:

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[17] Hewitt and Kenyon found 142° at 19 mm.

[18] The highest molecular rotation found by Hewitt and Kenyon is $[M]^{20}_{D} = 31.0°$.

7.920 mg. substance: 0.334 cc. N_2 at 763.8 mm. and 24°.
$C_{22}H_{23}O_2N$. Calculated. N 4.2.
Found. " 4.9.

The phenylurethane of this same carbinol was dextrorotatory in absolute alcohol.

$$[\alpha]_D^{23.5} = \frac{+0.83^\circ \times 100}{2 \times 5.28} = +7.9^\circ.$$

It was unfortunately very soluble in all organic solvents, even ligroin, and was not obtained pure. It melted at 40–41°. The nitrogen content by Dumas was too high by 1 per cent.

Levo-Ethyl-β-Cyclohexethyl Carbinol

Reduction of Levo-Ethylphenethyl Carbinol.—10 gm. of the pure carbinol ($\alpha_D^{25} = -12.95^\circ$) were dissolved in 90 cc. of pure glacial acetic acid and shaken with 0.2 gm. of Adams' PtO_2 catalyst with hydrogen under 3 atmospheres pressure. The hydrogen absorption was rapid and regular. After several hours, 0.3 gm. more catalyst was added. The absorption was now very rapid and regular but stopped suddenly. However, an analysis of the crude carbinol isolated, after thorough drying, showed that complete reduction had taken place. No hydrocarbon was found. The carbinol boiled at 103–108° at 0.2 mm. and had the following composition.

5.205 mg. substance: 14.835 mg. CO_2 and 6.095 mg. H_2O.
$C_{11}H_{22}O$. Calculated. C 77.7, H 12.9.
Found. " 77.7, " 13.1.

The carbinol had the following rotations.

Without solvent, 1 dm. tube, $\alpha_D^{24} = -3.89^\circ$, $[\alpha]_D^{24} = -4.32^\circ$, $D_4^{24} = 0.898$, $[M]_4^{24} = -7.34^\circ$.

$$\text{In absolute alcohol } [\alpha]_D^{24} = \frac{-1.07^\circ \times 100}{1 \times 21.20} = -5.0^\circ.$$

$$\text{In absolute ether } [\alpha]_D^{24} = \frac{-0.82^\circ \times 100}{1 \times 14.86} = -5.5^\circ.$$

α-Naphthylurethane of Levo-Ethyl-β-Cyclohexethyl Carbinol.—2 gm. of the pure carbinol were treated with 2.0 gm. of β-naphthyl isocyanate and the resulting mixture warmed for 2 hours on the steam bath. In 5 minutes it had already begun to crystallize. After cooling, the mass was washed with ligroin and recrystallized twice from 95 per cent alcohol. The pure white needles melted at 114–115° and analyzed as follows:

100.0 mg. substance required 2.91 cc. 0.1 N HCl (Kjeldahl).
$C_{22}H_{29}O_2N$. Calculated. N 4.1. Found. N 4.1.

The urethane had the following rotation.

$$\text{In absolute alcohol } [\alpha]_D^{22} = \frac{+0.06° \times 100}{1 \times 2.84} = +2.1°.$$

In chloroform the urethane was levorotatory.

$$[\alpha]_D^{22} = \frac{-0.61° \times 100}{1 \times 6.24} = -9.8°.$$

Ethylstyryl Ether

Reduction of the Levo-Ether of Levo-Ethylstyryl Carbinol.—The ether was not reduced by colloidal palladium. Thus when 2 gm. of the ether ($[\alpha]_D^{25} = -71.0°$) were shaken in the usual way with palladium, only a little hydrogen was absorbed after 7 hours, and the activity fell only to −66.8° at 26°.

It was however slowly reduced by Adams' PtO_2 in 95 per cent alcohol. When 1 gm. of the ether was thus reduced, the hydrogen absorption, though slow, was steady, and about the correct volume of hydrogen was absorbed. The ether, recovered in the usual way, and dried at 100° at 1.5 mm. analyzed as follows:

3.060 mg. substance: 9.490 mg. CO_2 and 2.720 mg. H_2O.
$C_{22}H_{30}O$. Calculated. C 85.2, H 9.7.
Found. " 84.6, " 9.9.

It had the following activity in absolute ether.

$$[\alpha]_D^{25} = \frac{-0.39° \times 100}{1 \times 8.06} = -4.8°.$$

This activity may well be too high or even dextro for the saturated ether was not entirely free from the unsaturated ether.

Attempts to make a solid tetrabromide of the unsaturated ether failed. The theoretical amount of bromine was absorbed without evolution of hydrogen bromide. The product was an oil, soluble in ligroin. It decomposed on standing.

Attempts to distil the unsaturated ether also resulted in decomposition.

Levo-Ethylcyclohexyl Carbinol

Reduction of Levo-Ethylphenyl Carbinol.—6 gm. of the carbinol ($\alpha_D^{23.5} = -25.68°$; $[\alpha]_D^{23.5} = -25.93°$[19]) dissolved in 50 cc. of glacial acetic acid were shaken with Adams' PtO_2 catalyst with hydrogen at 3 atmospheres pressure, until no more gas was absorbed. The platinum was then filtered off, the acid neutralized with sodium hydroxide, and the mixture extracted thoroughly with ether. After drying over anhydrous potassium carbonate the ether was pumped off and the remaining liquid distilled at 10 mm. About two-thirds came over at 39–40°. The remainder boiled at 80–85° at 10 mm. and had a strong camphor-like odor, very similar to methylcyclohexyl carbinol. In ether it had the following rotation.

$$[\alpha]_D^{21.5} = \frac{-1.39° \times 100}{1 \times 21.82} = -6.4°.$$

The carbinol was then redistilled and the fraction boiling at 85.5–86° at 9 mm. was collected. It had the following composition.

2.555 mg. substance: 7.180 mg. CO_2 and 3.405 mg. H_2O.
$C_9H_{18}O$. Calculated. C 76.1, H 12.7.
Found. " 76.6, " 13.2.

In a 50 mm. tube without solvent, it gave at 24° a levorotation of 2.93°; $\alpha_D^{24} = -5.86°$.

The liquid which constituted two-thirds of the reduction product, boiling at 39–40° at 10 mm., distilled at atmospheric pressure at 152–154°.[20] This fact and the following analysis identified the substance as propylcyclohexane.

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[19] Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, **99**, 45 (1911) give as a maximum $[\alpha]_D^{20} = 27.73°$.

[20] Sabatier and Senderens found 153–154° (Sabatier, P., and Senderens, J. B., *Compt. rend. Acad.*, **132**, 568 (1901)).

4.395 mg. substance: 13.775 mg. CO_2 and 5.595 mg. H_2O.
C_9H_{18}. Calculated. C 85.7, H 14.3.
Found. " 85.5, " 14.2.

Ethylbenzyl Carbinol

Resolution of Ethylbenzyl Carbinol.—Ethylbenzyl carbinol was easily resolved with strychnine. The salt, prepared in 95 per cent alcohol seemed at first to be more soluble in 80 to 85 per cent alcohol than in 95 per cent; but after several recrystallizations from 95 per cent alcohol this property apparently disappeared. After four such recrystallizations, the resulting phthalate (after hydrolysis) showed no increase in activity and had the following rotation in absolute alcohol.

$$[\alpha]_D^{25} = \frac{-1.17° \times 100}{1 \times 6.35} = -18.4°.$$

The carbinol obtained in the usual way boiled at 104–106° at 9 mm. and analyzed as follows:

3.365 mg. substance: 9.870 mg. CO_2 and 2.890 mg. H_2O.
$C_{10}H_{14}O$. Calculated. C 80.0, H 9.3.
Found. " 80.0, " 9.6.

In absolute ether it had the following activity.

$$[\alpha]_D^{27} = \frac{-2.90° \times 100}{1 \times 13.37} = -21.7°.$$

Without solvent, 1 dm. tube, $\alpha_D^{27} = -33.49°$, $D_4^{26} = 0.972$, $[M]_D^{27} = -51.7°$.

Levo-Ethylhexahydrobenzyl Carbinol

Reduction of Levo-Ethylbenzyl Carbinol.—5.5 gm. of the carbinol ($\alpha_D^{27} = -33.49°$) dissolved in 50 cc. of pure glacial acetic acid were shaken with hydrogen at 30 pounds pressure with 0.5 gm. of Adams' PtO_2 catalyst. The reduction was regular and complete in about 2 hours. The reduced carbinol isolated in the usual way was thoroughly dried at 0.8 mm. at 70° for about 1 hour. An analysis of this material was as follows:

3.425 mg. substance: 9.725 mg. CO_2 and 4.025 mg. H_2O.
$C_{10}H_{20}O$. Calculated. C 76.9, H 12.8.
Found. " 77.4, " 13.2.
Calculated for hydrocarbon $C_{10}H_{20}$. C 85.7, H 14.3.

These values indicate roughly that only 8 per cent of the carbinol was reduced to the corresponding hydrocarbon. The carbinol was then distilled. The first fraction up to 102° at 10 mm. consisted of both hydrocarbon and reduced carbinol. The second and main fraction, boiling point 102–103.5° at 10 mm., was pure ethylhexahydrobenzyl carbinol which analyzed as follows:

3.660 mg. substance: 10.300 mg. CO_2 and 4.290 mg. H_2O.
$C_{10}H_{20}O$. Calculated. C 76.9, H 12.8.
Found. " 76.7, " 13.1.

In absolute ether the carbinol had the following activity.

$$[\alpha]_D^{23} = \frac{-5.08° \times 100}{1 \times 20.06} = -25.3°.$$

Without solvent, 1 dm. tube, $\alpha_D^{24.5} = -24.30°$, $D_4^{24} = 0.900$, $[M]_D^{24.5} = -42.1°$.

Dextro-Isopropylcyclohexyl Carbinol

Reduction of Levo-Isopropylphenyl Carbinol.—5.5 gm. of the carbinol ($\alpha_D^{23.5} = -9.01°$) boiling at 105–108° at 14 mm. dissolved in an alcoholic solution of glacial acetic acid (1:1) were shaken with hydrogen at 30 pounds pressure with 0.5 gm. of Adams' PtO_2 catalyst until no more hydrogen was absorbed. The carbinol was recovered in the usual way and distilled *in vacuo*. At 9 mm. there was a considerable amount of a liquid boiling at 47–48° probably the saturated hydrocarbon. The pure reduced carbinol boiled at 91–94° at 9 mm. and amounted to 2.3 gm. or about a 40 per cent yield. It had a characteristic camphor-like odor and gave the following analysis.

3.520 mg. substance: 9.945 mg. CO_2 and 3.945 mg. H_2O.
$C_{10}H_{20}O$. Calculated. C 76.9, H 12.8.
Found. " 77.0, " 12.6.

In the homogeneous state, 1 dm. tube, the carbinol was dextrorotatory, $\alpha_D^{23.5} = +2.75°$.

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THE HYDROGENATION OF UNSATURATED LACTONES TO DESOXY ACIDS

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During the past year, by a rather curious coincidence, reports of similar experiences have appeared from four different laboratories where work was carried out in distinct fields. These have had to do with the cleavage of certain lactones to desoxy acids on catalytic hydrogenation. The first of these was the report of Borsche and Peitzsch[1] on the catalytic reduction of methysticin to tetrahydromethysticinic acid through the intermediate lactone dihydromethysticin. Simultaneously there appeared a report of Mannich and Butz[2] on the hydrogenation of several unsaturated δ-lactones, which led through the intermediate saturated lactone to the desoxy acid. Somewhat later La Forge and Smith[3] described their observations on the hydrogenation products of rotenone. The latter was found to yield on addition of 2 atoms of hydrogen principally two isomeric substances, a neutral dihydrorotenone and an acid, rotenonic acid. In the latter case the acid was produced by cleavage of the lactone group to the desoxy acid and appeared to be independent of the hydrogenation of the double bond.[4]

Before the appearance of the above reports Jacobs and Gustus had already met with and later published[5] a similar experience in the case of the hydrogenation with various catalysts of the unsaturated lactone anhydro-γ-isogitoxigenic methyl ester to the saturated desoxy acid, γ-digitoxanoldiacid monomethyl ester following the absorption of 2 mols of hydrogen. In this case all efforts to intercept the reaction at the 1 mol stage in order to obtain the saturated lactone by hydrogenation of the double bond alone failed. The only product of the reaction observed was the saturated desoxy acid and unchanged starting material. Since in the case of a somewhat analogous saturated lactone, γ-isodigitoxigenic acid (methyl ester), all attempts to accomplish a similar lactone cleavage had failed, the unique behaviour of the unsaturated lactone mentioned above suggested along with other previously discussed observations that the double bond in this substance is associated with the lactone ring. In view of the importance of this conclusion for the determination of certain points in the structure of gitoxigenin, it was decided to investigate the behavior of simpler unsaturated lactones on catalytic hydrogenation. This study has yielded rather interesting results.

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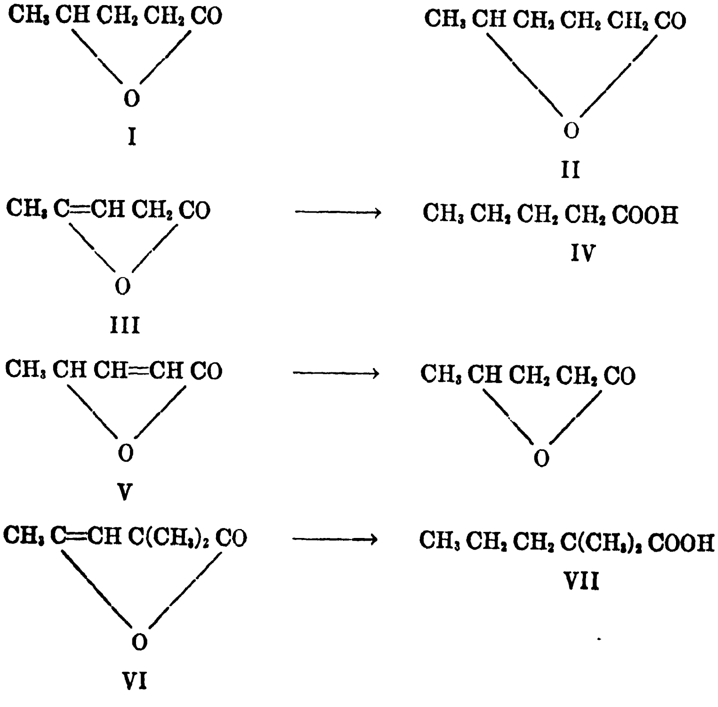
[1] Borsche, W., and Peitzsch, W., *Ber. chem. Ges.*, **62**, 360 (1929).

[2] Mannich, C., and Butz, A., *Ber. chem. Ges.*, **62**, 461 (1929).

[3] La Forge, F. B., and Smith, L. E., *J. Am. Chem., Soc.*, **51**, 2574 (1929); **52**, 1088 (1930).

[4] The cleavage of the furane ring in another type of substance on catalytic hydrogenation has been presented by Kaufmann, W. E., and Adams, R., *J. Am. Chem. Soc.*, **45**, 3029 (1923).

[5] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **86**, 203 (1930).



I

II

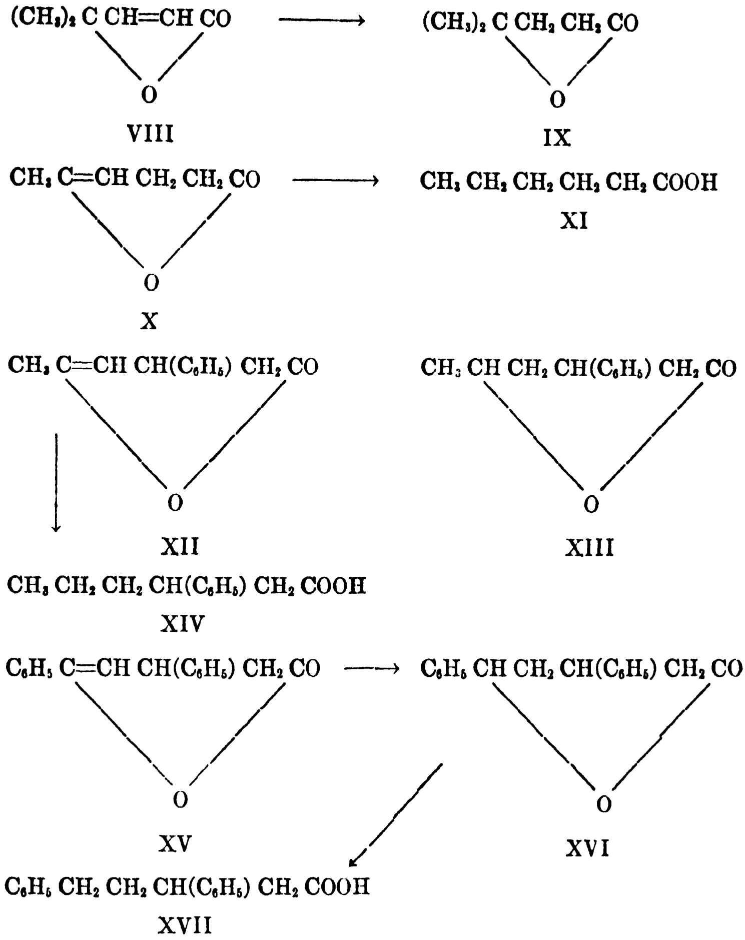
III

IV

V

VI

VII



A comparison of the behaviors of $\Delta^{\beta,\gamma}$- and $\Delta^{\alpha,\beta}$-angelicalactone has shown a striking contrast. In repeated experiments $\Delta^{\beta,\gamma}$-angelicalactone (Formula III) was found to absorb 2 mols of catalytically activated hydrogen very readily with the formation of valeric acid (Formula IV). On the other hand, the $\Delta^{\alpha,\beta}$-isomer (Formula V) was found to take principally a different course and stopped at approximately the 1 mol stage with the production of γ-valerolactone (Formula I). In this case only approximate figures for 1 mol were obtained and in different experiments a varying amount of excessive absorption was noted with a corresponding formation of valeric acid. But this excessive absorption occurred during the main reaction and once prompt absorption had stopped, no further appreciable consumption of hydrogen could be detected. The relatively small formation of valeric acid, we believe, is attributable to the presence of the $\Delta^{\beta,\gamma}$- isomer. It is well known that the two isomers readily undergo interconversion during certain reactions. During the hydrogenation a portion of the $\Delta^{\alpha,\beta}$-angelicalactone must rearrange to the $\Delta^{\beta,\gamma}$form and in an amount which may vary with temperature conditions and the rapidity with which hydrogenation proceeds. Repeated attempts to cause such a cleavage of γ-valerolactone itself to valeric acid were unsuccessful. In such experiments subsequent addition of $\Delta^{\beta,\gamma}$-angelicalactone to the mixture resulted in prompt quantitative hydrogenation of the latter to valeric acid and the unattacked valerolactone was readily recovered as such. In view of the comparative instability of $\Delta^{\beta,\gamma}$-angelicalactone and its ready saponification to levulinic acid, it appeared possible that its peculiar behavior might be due to the intermediate formation of the keto acid which might be reduced to valeric acid. This seemed excluded, however, because in a direct study of the behaviour of levulinic acid itself under the conditions of hydrogenation, it was found to absorb hydrogen very slowly and in a manner not comparable with $\Delta^{\beta,\gamma}$-angelicalactone.

In order to make more clear cut the difference in behavior of the two types of unsaturated γ-lactones we have selected two substances in which the double bond is fixed without the possibility of a shift within the lactone ring, namely α,α-dimethyl-$\Delta^{\beta,\gamma}$-angelicalactone (Formula VI) and γ-methyl-$\Delta^{\alpha,\beta}$-angelicalactone (Formula VIII). In a former case a prompt hydrogenation to the desoxy acid, α,α-dimethyl-*n*-valeric acid occurred while in the case of the $\Delta^{\alpha,\beta}$-lactone only 1 mol of hydrogen was absorbed with the formation of γ-methyl-γ-valerolactone. We have then turned to the δ-lactones with similar results. As in the case of γ-valerolactone, all attempts to cleave δ-*n*-caprolactone were unsuccessful. On the other hand, the lactone of δ-hydroxy-Δ-$^{\gamma,\delta}$ hexenoic acid (Formula X) promptly absorbed 2 mols of hydrogen with the formation of *n*-caproic acid.

From these results it appears that unsaturated lactones in which the double bond is attached to the point of lactonization, as in the lactones of enolized keto acids, are reduced to the desoxy acid on catalytic hydrogenation. The results of Mannich and Butz with the lactone of δ-hydroxy-β, δ-diphenyl-$\Delta^{\gamma,\delta}$-pentenoic acid (Formula XV) and an analogous β-(methylenedioxyphenyl) derivative are in conformity with these results. However, their experience with the lactone of δ-hydroxy-β-phenyl-$\Delta^{\gamma,\delta}$-hexenoic acid (Formula XII) appeared to present an exception. Since they have reported that their substance failed to absorb hydrogen at all, it appeared to us that the failure might be attributable to manipulative complications rather than to the structure of the lactone. We have therefore reexamined the behavior of this substance using a different solvent and catalyst and found, contrary to Mannich and Butz, that it readily absorbs 2 mols of hydrogen with the formation of a desoxy acid and therefore presents a normal behavior. Their suggestion is, therefore, superfluous, that in the case of the phenyl-substituted unsaturated lactones which may be regarded as derivatives of substituted benzyl alcohols their hydrogenation to the desoxy acids is analogous to the reduction of benzyl alcohol to toluene. In view of the observation of these workers, however, that the hydrogenation of the diphenyl lactone (Formula XV) when interrupted at the 1 mol stage gives a good proportion of the saturated lactone (Formula XVI), which was therefore unquestionably the intermediate stage in the formation of the desoxy acid, it was of interest to determine whether the same situation would be met in the case of the hexenoic lactone (Formula XII). This was found, however, not to be the case at least by our procedure, since on interruption of the hydrogenation at the 1 mol stage about half of the reaction product proved to be the desoxy acid, and an approximately corresponding amount of starting material was found to be unattacked. If the saturated lactone was formed in this case as the intermediate stage, the amount in which it persisted was so small as to make its certain identification difficult. In the present studies we have consistently employed the same procedure throughout, namely alcohol as solvent and the platinum oxide of Adams and Shriner as catalyst. This procedure differed from that used by Mannich and Butz and the question is left open for the present whether the difference in behaviors of the diphenyl lactone (XV), as reported by them, and of the hexenoic lactone (XII) in our hands is attributable to this difference in procedure or to the substances themselves.

In the case of methysticin, according to Borsche and Peitzsch, the principal reaction is a prompt hydrogenation to dihydromethysticin, but accompanying this there is a simultaneous formation of about 10 per cent of the desoxy acid. They have apparently shown that the latter proceeds through the dihydro stage by the fact that dihydromethysticin may be transformed also, although far more slowly, into the desoxy acid. They have suggested as a possible explanation of the greater speeds of acid formation in the former case that the free energy developed during hydrogenation of the double bond of methysticin may accelerate the otherwise slow tendency to further reductive cleavage of the lactone ring. It is possible that this principle may more strongly govern in the case of lactones of enolized keto acids. Here the double bond is attached to the same carbon atom upon which lactonization has occurred so that the second reaction, *i.e.* cleavage to the desoxy acid, may be so accelerated as to keep pace with hydrogenation of the double bond. This is so marked that the saturated lactone which might be formed could not be readily isolated as such. This was substantiated by an experiment in which on hydrogenation of a mixture of $\Delta^{\beta,\gamma}$-angelicalactone and $^{\gamma}$-valerolactone the hydrogen absorbed and the acidity developed corresponded quantitatively with the amount of the former, and valerolactone was recovered unchanged. It is apparent that in certain cases, such as the lactones of Mannich and Butz, the factors involved are not so simple. Here the intermediate saturated lactones in appreciable amounts may be isolated as such, and may be in turn easily cleaved to the desoxy acids.

In summing up our experience, it appears that wherever a substance containing a lactone group and a double bond is promptly hydrogenated to the saturated desoxy acid without a definite break at the 1 mol stage with formation of the saturated lactone it may be taken as strongly presumptive evidence that one is dealing with the lactone of an enolized oxo-acid. These observations support the interpretation given in our former work to the behavior on hydrogenation of anhydro-γ-isogitoxigenic methyl ester where the saturated lactone γ-isogitoxigenic methyl ester was unattacked by catalytically activated hydrogen. The former is possibly a $\Delta^{\gamma,\delta}$ unsaturated δ-lactone.

There is, however, a point which requires further investigation, namely the behavior of the cardiac aglucones themselves on hydrogenation. In previous reports from this laboratory and from those of Windaus and Cloetta of experiments on hydrogenation, the formation only of the neutral saturated lactones has been noted. The possible production of any acid products has not been suggested. Since we have found that these substances are $\Delta^{\beta,\gamma}$ unsaturated γ-lactones, it is strange that their hydrogenation has not continued to the desoxy acids. This problem is being studied further in order to determine, if possible, the reason for such an apparently anomalous behavior.

EXPERIMENTAL

In the following experiments a constant procedure was used in which the substances were hydrogenated in ethyl alcoholic solution with platinum oxide catalyst prepared according to Adams and Shriner.

Hydrogenation of $\Delta^{\beta,\gamma}$*-Angelicalactone.*—This lactone was prepared by the method of Thiele, Tischbein, and Lossow[6] and a middle fraction which gave a b.p. of 55–56° at 12 mm. pressure was employed. A solution of 0.71 gm. of this fraction in ethyl alcohol was treated with 0.05 gm. of platinum oxide catalyst and shaken with hydrogen. The absorption of hydrogen proceeded rapidly and came to a stop after 15 minutes. However, observation was continued for several hours but no appreciable additional absorption could be detected. After correcting for the reduction of the catalyst the observed hydrogen absorption was found to be 325 cc. (760 mm., 0°).[7] Calculated for 2 mols of H_2, 325 cc. The reaction mixture smelled strongly of valeric acid and was acid in reaction. In this experiment the substance was not isolated.

In a second experiment under identical conditions, 1.05 gm. of $\Delta^{\beta,\gamma}$-angelicalactone with 0.05 gm. of catalyst were found to absorb 487 cc. of hydrogen within 30 minutes. Calculated for $2H_2$, 480 cc.

The free acid present in the alcoholic reaction mixture after filtering from catalyst was determined by direct titration against phenolphthalein with 0.1 N sodium hydroxide solution. Calculated for 100 per cent conversion to valeric acid, 107 cc. Found, 97 cc.

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[6] Thiele, J., Tischbein, R., and Lossow, E., *Ann. Chem.*, 319, 184 (1901).

[7] All gas volumes are given for the dry gas at 0° and 760 mm.

The presence of valeric acid was confirmed by the preparation of the silver salt. For this purpose the alkaline mixture was concentrated under diminished pressure to remove the alcohol. The solution of the residue in a few cc. of water was carefully neutralized with dilute sulfuric acid and was then treated with 10 per cent silver nitrate solution. Recrystallization of the salt from water gave white needles which were dried for analysis at 60° and 15 mm. over $CaCl_2$.

5.776 mg. substance: 2.270 mg. H_2O, 6.124 mg. CO_2, 2.966 mg. Ag.
$C_5H_9O_2Ag$. Calculated. C 28.71, H 4.35, Ag. 51.63.
Found. " 28.91, " 4.39, " 51.35.

Hydrogenation of $\Delta^{\alpha,\beta}$-Angelicalactone.—The $\Delta^{\alpha,\beta}$-angelicalactone (b.p. 84.5°, 12 mm.) in 0.2 per cent solution did not reduce ammoniacal silver solution. 3.21 gm. were hydrogenated with 0.06 gm. of platinum oxide. The reaction definitely stopped after 74 minutes. The calculated absorption for 1 mol of H_2 is 733 cc. Found, 795 cc.

That this excessive absorption over the 1 mol requirement indicated the formation of a small amount of valeric acid was confirmed by the titration experiments. Direct titration against phenolphthalein showed the presence of free acid corresponding to 5.07 cc. of N sodium hydroxide solution. 50 cc. of N alkali were then added and after refluxing for 1 hour the mixture was titrated back. The additional alkali consumed by saponification of valerolactone was 27.5 cc. of N sodium hydroxide. The total calculated alkali requirement is 32.8 cc. Found, 32.6 cc.

The formation of γ-valerolactone was confirmed by its isolation as follows. The above alkaline titration mixture was concentrated to dryness. A concentrated solution of the residue was strongly acidified to Congo red with dilute sulfuric acid, and the solution was refluxed for 30 minutes to insure lactonization. After extraction with ether and washing the extract with 50 per cent potassium carbonate solution the extract was dried with ignited potassium carbonate. Fractionation gave γ-valerolactone which boiled at 16 mm. at 87–90°.

4.093 mg. substance: 2.940 mg. H_2O, 8.986 mg. CO_2.
$C_5H_8O_2$. Calculated. C 59.96, H 8.06.
Found. " 59.88, " 8.04.

The course of the hydrogenation was verified by a second experiment conducted in a similar manner. The fact that the lactone had been permitted to stand for 8 days before use with the possibility of appreciable isomerization probably accounts for the somewhat larger relative absorption of hydrogen beyond the 1 mol stage with a corresponding formation of a greater amount of valeric acid.

2.99 gm. of lactone and 0.05 gm. of catalyst were used. Calculated absorption for 1 mol of H_2, 683 cc. Found, 817 cc.

5.3 cc. of N sodium hydroxide solution were required to neutralize the free acidity and an additional 24.7 cc. for saponification or a total of 30 cc. Calculated, 30.5 cc.

The γ-valerolactone recovered boiled at 90–91° at 16 mm.

5.947 mg. substance: 4.315 mg. H_2O, 13.050 mg. CO_2. Found. C 59.85, H 8.11.

Attempted Hydrogenation of γ-Valerolactone.—1.28 gm. of γ-valerolactone, b.p. 96–100° at 29 mm., in ethyl alcoholic solution were shaken with hydrogen and 0.05 gm. of catalyst. After the prompt reduction of the latter, no further absorption of hydrogen was noted even after 20 hours, although an additional portion of catalyst had been added after the 1st hour. Subsequent addition of 0.59 gm. of $\Delta^{\beta,\gamma}$-angelicalactone to the mixture resulted in a prompt addition within 3 hours of 2 mols of H_2 based on the angelicalactone used. Found, 264 cc. Calculated, 270 cc.

In a second experiment a mixture of the two lactones was used at the start. 2.16 gm. of γ-valerolactone and 0.71 gm. of $\Delta^{\beta,\gamma}$-angelicalactone were treated in the usual manner with 0.05 gm. of catalyst. Hydrogenation ceased abruptly at the end of 30 minutes. The observed hydrogen absorption was 315 cc. Calculated for the $\Delta^{\beta,\gamma}$-angelicalactone present, 324 cc.

Titration of the free acid in the reaction mixture confirmed the conversion of the unsaturated lactone to valeric acid, while the saturated lactone remained unchanged. Direct titration required 6.6 cc. of N sodium hydroxide solution. Calculated for valeric acid formed from 0.71 gm. of angelicalactone, 7.2 cc. The titration mixture was then boiled with an excess of N alkali and again titrated back. Calculated for the valerolactone originally used, 21.6 cc. Found, 20.5 cc.

The titration mixture after concentration was relactonized as previously given and γ-valerolactone was recovered. It boiled at 17 mm. at 86–87°.

5.910 mg. substance: 4.380 mg. H_2O, 12.920 mg. CO_2. Found. C 59.62, H 8.29.

Hydrogenation of α,α-Dimethyl-$\Delta^{\beta,\gamma}$Angelicalactone.—This lactone, which was prepared according to Pinner,[8] boiled at 18 mm. at 59° and formed long, transparent prisms when cooled in ice.

0.330 gm. of the lactone with 0.015 gm. of catalyst absorbed within 22 minutes 118 cc. of hydrogen. Calculated for $2H_2$, 117 cc.

In another experiment, 1.69 gm. of lactone and 0.05 gm. of catalyst were employed. For some undetermined reason (possibly due to poisoning of the catalyst), the absorption of hydrogen was unusually slow. This came to a stop after 18 hours. The observed absorption was 591 cc. Calculated for $2H_2$, 601 cc.

The filtered reaction mixture on titration required for neutralization 11.5 cc. of N sodium hydroxide solution. The calculated requirement was 13.4 cc. From the concentrated solution the silver salt of α,α-dimethylvaleric acid was prepared which formed needles after recrystallization from water.

6.430 mg. substance: 3.235 mg. H_2O, 8.367 mg. CO_2, 2.923 mg. Ag.
$C_7H_{13}O_2Ag$. Calculated. C 35.45, H 5.54, Ag 45.53.
Found. " 35.50, " 5.63, " 45.46.

Hydrogenation of γ-Methyl-$\Delta^{\alpha,\beta}$-Angelicalactone.—Terelactone was prepared from the dibromide of pyroterebic acid by boiling with aqueous sodium carbonate solution. The pyroterebic acid necessary as an intermediate was readily obtained from its isomer by the method of Goldberg and Linstead[9] in which $\Delta^{\alpha,\beta}$-isohexenoic acid is prepared by the Doebner reaction and converted to the isomer, pyroterebic acid, by heating with alkali. The terelactone was a colorless, neutral liquid which boiled at 72 mm. at 127°.

0.59 gm. of terelactone and 0.05 gm. of catalyst showed a complete absorption of 1 mol of hydrogen within 7 minutes. On continued shaking for 20 hours more, no appreciable additional absorption was noted. The observed absorption was 107 cc. Calculated for 1 mol of H_2, 118 cc.

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[8] Pinner, A., *Ber. chem. Ges.*, 15, 579 (1882).

[9] Goldberg, A. A., and Linstead, R. P., *J. Chem. Soc.*, 2354 (1928).

The free acid in the mixture was not appreciable and only 0.19 cc. of N alkali was required for neutralization. On saponification with an excess of alkali an additional 4.9 cc. of N alkali were consumed, or a total of 5.1 cc. Calculated, 5.3 cc. The initial slight acidity was possibly due to slight, spontaneous opening of the lactone to the hydroxy acid. From the above saponification mixture after concentration the silver salt of γ-hydroxyisocaproic acid was prepared in the usual manner and formed lustrous needles from water.

5.640 mg. substance: 2.380 mg. H_2O, 6.223 mg. CO_2, 2.530 mg. Ag.

$C_6H_{11}O_3Ag$.	Calculated.	C 30.13,	H 4.64,	Ag 45.15.
	Found.	" 30.09,	" 4.72,	" 44.86.

Hydrogenation of the Lactone of δ-Hydroxy-$\Delta^{\delta,\gamma}$-Hexenoic Acid.—This lactone was prepared by the method of Vorländer and Knötzsch[10] and showed a b.p. of 112–115° at 69 mm.

0.625 gm. of the lactone and 0.05 gm. of catalyst gave a completed reaction corresponding to 2 mols of H_2 in 30 minutes. The absorption was 242 cc. Calculated, 250 cc.

On direct titration of the free acidity 5.2 cc. of N alkali were required. Calculated, 5.6 cc. The silver salt of caproic acid was prepared in the usual manner.

6.190 mg. substance: 2.800 mg. H_2O, 7.370 mg. CO_2, 2.990 mg. Ag.

$C_6H_{11}O_2Ag$.	Calculated.	C 32.39,	H 4.98,	Ag 48.39.
	Found.	" 32.47,	" 5.06,	" 48.30.

Hydrogenation of the Lactone of δ-Hydroxy-β-Phenyl-$\Delta^{\gamma,\delta}$-Hexenoic Acid.—This lactone was prepared by the method of Vorländer and Knötzsch[10] and showed a b.p. of 167–170° at 12 mm. 0.865 gm. was hydrogenated with 0.1 gm. of catalyst. After 20 minutes the reaction showed a decided change in the rate of absorption and then proceeded slowly. After an additional 20 minutes the reaction was interrupted since the continued slow absorption was probably due to reduction of the benzene nucleus. The observed absorption was 269 cc. Calculated for 2 mols of

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[10] Vorländer, D., and Knötzsch, A., *Ann. Chem.*, **294**, 317 (1897).

H_2, 206 cc. To neutralize the free acid developed, 37 cc. of N alkali were required. On subsequent saponification with excess alkali an additional 8 cc. were consumed or a total of 45 cc. possibly due to some ester formation during the hydrogenation. The calculated alkali equivalent for the original lactone is 47 cc.

The silver salt of the resulting β-phenyl-*n*-caproic acid was obtained as usual for its identification.

4.925 mg. substance: 2.390 mg. H_2O, 8.720 mg. CO_2, 1.780 mg. Ag.
$C_{12}H_{15}O_2Ag$. Calculated. C 48.16, H 5.05, Ag 36.09.
Found. " 48.29, " 5.43, " 36.15.

In another experiment the hydrogenation was interrupted after an alcoholic solution of 8.5 gm. of lactone with 0.05 gm. of catalyst had absorbed 1013 cc. or 1 mol of H_2. The alcoholic solution after filtration from catalyst was concentrated, the residue was taken up in ether, and the solution was extracted with aqueous potassium carbonate. Ether extraction of the acidified carbonate solution gave on concentration 4.5 gm. of crude acid as an oil. On fractionation the major portion distilled at 179–181° at 20 mm. and was found by analysis to be β-phenyl-caproic acid.

3.080 mg. substance: 2.305 mg. H_2O, 8.468 mg. CO_2.
$C_{12}H_{16}O_2$. Calculated. C 74.96, H 8.38.
Found. " 74.98, " 8.37.

The above ether solution containing the neutral fraction was concentrated and the residue, which consisted mostly of unchanged, unsaturated lactone, was converted into the acid by saponification with an excess of aqueous N sodium hydroxide solution. After reacidifying strongly with hydrochloric acid and refluxing for 30 minutes to relactonize any β-phenyl-δ-hydroxy-caproic acid which might have been present, the mixture was again extracted with ether. The β-phenyl-δ-keto-caproic acid which could not relactonize under these conditions was recovered by extraction of the ether solution with carbonate. Acidification of the carbonate solution gave 3.5 gm. of the keto acid in characteristic needles which melted at 83°.

The neutral material which remained after the above carbonate extraction of the keto acid and concentration of the ether solution weighed approximately 0.2 gm. This was an amount too small for direct purification for analysis. An attempt to prepare a silver salt after saponification was unsuccessful. The possibility remains, however, that this small amount of material may have been saturated β-phenyl-δ-caprolactone which may represent the intermediate stage in the formation of the desoxy acid or may have been formed from a small amount of an isomer of the original unsaturated lactone in which the double bond has shifted from the position γ, δ.

[Reprinted from SCIENCE, May 2, 1930, Vol. 71, No. 1844, pp. 463–464]

AN ELECTROMAGNETIC PUMP

BY HEINZ ROSENBERGER

(From the Laboratories of The Rockefeller Institute for Medical Research)

During the course of some investigations in this laboratory, it became necessary to devise a pump which could be used to circulate sterile fluid in a system free from any rubber, metal, oil, grease or cement. To fulfil these requirements, a pump has been constructed entirely of glass, in which the motion of the piston is actuated by electromagnetic forces.

The diagram shows the pump in cross section. The pump cylinder (1) is a glass tube which has a carefully ground valve (2) at its lower end. The piston (3) consists of two tubes with a soft iron core fused between them. The lower end of the piston has a valve (4) which is identically the same as the cylinder valve (2). Both valves close by gravity. The pump cylinder is surrounded by a lower solenoid (5) and an upper solenoid (6). Both solenoids are hooked up in separate circuits. By means of a three-pole, automatic, mercury switch actuated by a rocking device, the electric current flows through the solenoids periodically in such a way that they are switched on and off, one after the other, with an intermediate state in which both solenoids are magnetized for a short time. Thus, a magnetic field is created inside the solenoids. The center of this field travels up and down periodically.

The iron core inside of the piston is attracted into the center of the magnetic field. The piston moves up and down continually, like the plunger of any pump. The up-stroke of the piston opens the cylinder valve (2) and closes the piston valve (4), while the down-stroke closes the cylinder valve and opens the piston valve. By this means, any fluid can be circulated or transferred.

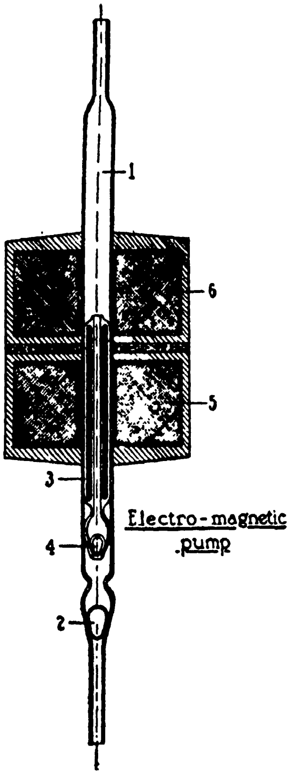

FIG. 1

The pump has many practical applications, especially where it is necessary to maintain sterility. It can also be employed for blood, and for strong acids, alkalis or other dangerous fluids.

[Reprinted from THE JOURNAL OF GENERAL PHYSIOLOGY, May 20, 1930,
Vol. 13, No. 5, pp. 547–552]

SALT BRIDGES AND NEGATIVE VARIATIONS

BY W. J. V. OSTERHOUT AND S. E. HILL

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Accepted for publication, March 10, 1930)

If a cell of *Nitella* imbibed with tap water be stimulated at such a point as *A* (Fig. 1) a negative variation usually passes to *B*, *D*, and *C*. If sufficient chloroform be applied at *D* the negative variation fails[1] to appear at *C* and we therefore speak of the chloroformed spot as a block.

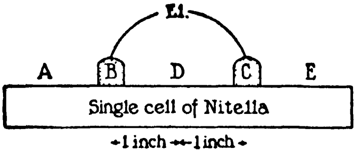

FIG. 1

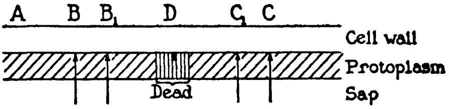

FIG. 2

FIG. 1. Diagram to show arrangement of experiments.

FIG. 2. Hypothetical diagram showing electrical conditions in the protoplasm. The arrows indicate E.M.F. (the direction shows how the positive current tends to flow). The protoplasm at *D* is shaded vertically to indicate that it has been killed by chloroform; it is assumed that it has been previously treated as in Fig. 4 to prevent negative variations from originating at *D*.

According to the local circuit theory[2] we should expect a variation starting at *A* to bring about a variation at *C* if we put a salt bridge around the chloroformed spot. For if the cell is imbibed with tap water (or 0.001 M KCl) we may diagram the protoplasm as in Fig. 2 and assume that a negative variation traveling from *A* to *B* makes an outward flow of current[3] at *B* causing[2c] the E.M.F. at *B* to approach zero and a flow

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[1] It is necessary to arrange the experiments so that no negative variation originates at *D*. See footnote 2 *c* and Fig. 4.

[2] *Cf.* (*a*) Lillie, R. S., Protoplasmic action and nervous action, University of Chicago Press, Chicago, 1923; (*b*) Davis, H., *Physiol. Reviews*, 1926, **6**, 547; (*c*) Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1929–30, **13**, 391.

[3] By this is meant the sort of flow indicated by the arrow at B_1 in Fig. 3.

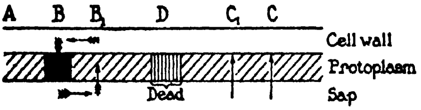

FIG. 3

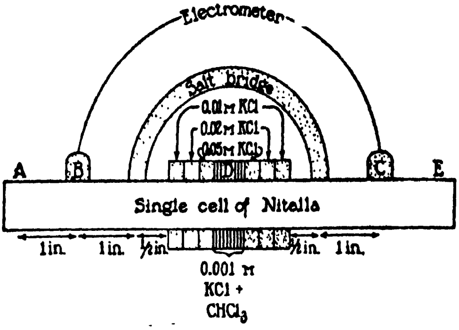

FIG. 4

FIG. 3. As in Fig. 2 but showing a flow of current between *B* and B_1 (flow is indicated by feathered arrows, E.M.F. by plain arrows). The protoplasm at *B* is shaded horizontally to indicate that it has temporarily lost its potential (that at *D* is shaded vertically to indicate that it is dead).

FIG. 4. Diagram to show arrangement of experiments. The protoplasm at *D* has been killed by 0.001 M KCl saturated with chloroform and various concentrations of KCl are placed on each side to prevent negative variations[2e] from originating at *D* (and passing along the cell in either direction).

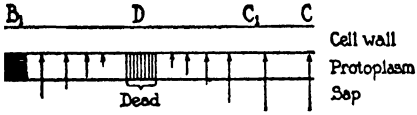

FIG. 5

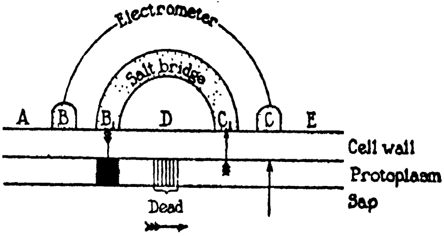

FIG. 6

FIG. 5. As in Fig. 4 but without a salt bridge: the protoplasm at B_1 has lost its potential but the P.D. gradient is not steep enough to cause sufficient outward flow at C_1 to start a variation. The arrows indicate E.M.F.

FIG. 6. As in Fig. 5 but with a flow of current through a salt bridge as indicated by the feathered arrows (the horizontal flow in the protoplasm and cell wall is relatively small and is therefore not indicated). Plain arrows indicate E.M.F. and feathered arrows indicate current.

to start between *B* and B_1 as shown in Fig. 3. This in turn will cause the E.M.F. at B_1 to approach zero and in this way the variation will travel along the cell.[4]

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[4] *Cf.* Blinks, L. R., Harris, E. S., and Osterhout, W. J. V., *Proc. Soc. Exp. Biol. and Med.*, 1928–29, 26, 836.

The situation when a block is placed in the center (as in Fig. 4 but without the salt bridge) probably resembles that shown in Fig. 5. The P.D. gradient is not steep enough to permit sufficient outward flow at C_1 to reduce its E.M.F. to zero and start a negative variation.

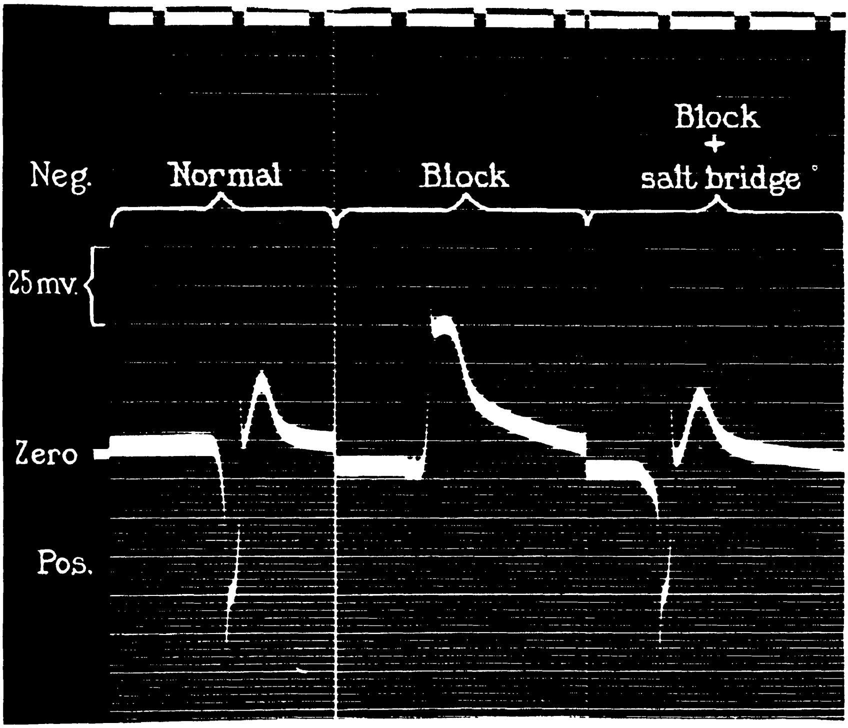

FIG. 7. Photographic record showing the P.D. of *B* in relation to *C*.

The part marked "normal" shows a diphasic action current, the experiment being arranged as in Fig. 1 (with 0.001 M KCl at *B* and *C*); the cell is stimulated at *E* (by applying 0.05 M KCl[2c]) and the negative variation reaching *C* makes *B* appear positive because we are recording the P.D. of *B* with reference to *C*; afterward it reaches *B* making it appear negative.

The part marked "block" shows the effect of stimulation at *A* when the experiment is arranged as in Fig. 4 but without a salt bridge. A negative variation starting at *A* reaches *B* but since it cannot pass *D* the action current is monophasic.

The part marked "block plus salt bridge" shows the action current produced when the experiment is arranged as in Fig. 4 (with salt bridge). The action current produced by stimulation at *E* is diphasic like the one labelled "normal" (at the start of the record) for reasons given in the text.

The time marks represent 5-second intervals.

But if we put a salt bridge[5] between B_1 and C_1 and the E.M.F. at B_1 falls to zero (as the result of a negative variation) a flow can take place as shown in Fig. 6, which will reduce the E.M.F. at C_1 to zero, starting a negative variation.[6] We thus obtain the record shown in Fig. 7.

The experiment referred to in Fig. 7 was arranged as in Fig. 4, putting a piece of cotton soaked in 0.001 M KCl saturated with chloroform in the middle of the cell

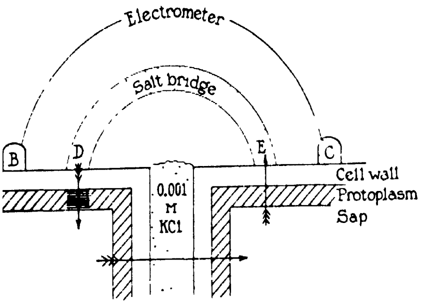

FIG. 8

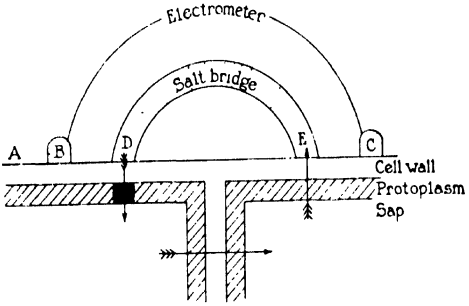

FIG. 9

FIG. 8. Hypothetical diagram of electrical conditions in two cells (from different plants) in contact with cotton soaked with 0.001 M KCl and connected by a salt bridge. The protoplasm at *D* has lost its potential (as indicated by the horizontal shading) and in consequence there is a flow of current between *D* and *E* as indicated by the feathered arrows. (Since the horizontal flow in the horizontal cell walls and the vertical flow in the vertical cell walls is relatively small it is not indicated.)

FIG. 9. Hypothetical diagram of electrical conditions in two cells in their natural union: they are connected by a salt bridge. The protoplasm at *D* has lost its potential as indicated by the horizontal shading and in consequence there is a flow of current as indicated by the feathered arrows (since the horizontal flow in the horizontal cell walls and the vertical flow in the vertical cell walls is relatively small it is not indicated).

and on each side pieces of cotton soaked in various concentrations of KCl (as shown in the figure) so arranged as to make a gradual gradient (this, as shown in a previous paper, prevents the chloroformed spot from starting negative variations). At *B* and *C* 0.001 M KCl was applied and 0.05 M KCl was placed at *A* or *E* to start a series of negative variations.

The salt bridge was composed of cotton soaked in 0.001 M KCl in all cases.

All experiments were performed on *Nitella flexilis* at 19° to 20°C. the technique being that described in previous papers unless otherwise stated.

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[5] Two calomel electrodes connected by a wire may serve.

[6] This would, of course, travel in both directions.

In view of this result it does not seem strange that a salt bridge enables a negative variation to set up a similar variation even in a cell taken from another plant and placed beside it at a distance of half an inch or more, as shown[7] in Fig. 8. In this case the circuit passes 4 times through living protoplasm (instead of twice, as in the preceding experiments). Since the resistance of the protoplasm is very high[8] we might expect the flow of current to be greatly reduced but in spite of this a negative variation starting at *A* and reaching *D* is promptly followed by one in the other cell when the salt bridge has a sufficiently low resistance (*e.g.*, cotton soaked in 0.001 M KCl).

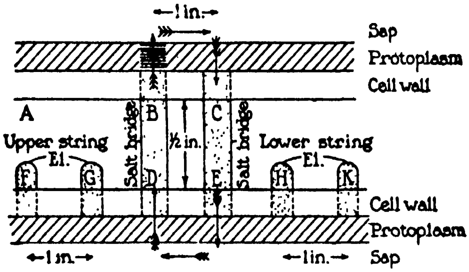

FIG. 10. Hypothetical diagram of electrical conditions in two cells (from different plants) connected by two salt bridges. The protoplasm at *B* has lost its potential (as indicated by the horizontal shading) and in consequence there is a flow of current as indicated by the feathered arrows. Since the horizontal flow in the protoplasm and in the horizontal cell walls is relatively small it is not indicated.

If an experiment be arranged as in Fig. 9, employing two cells in their natural union, the negative variation starting at *A* and reaching *D* can start a variation at *E* by the aid of a salt bridge but this happens much less frequently when the salt bridge is absent: apparently the cell wall may act as a salt bridge in some cases, as was observed by Mr. Harris and previously reported.[4]

A positive result is also obtained when the experiment is arranged as in Fig. 10. In this case the currents would presumably be as indicated so that in the lower cell there would be an outward current at *D* which could stimulate. This is commonly the case. Preliminary experiments of this sort were carried out by Mr. Harris and have been previously reported.[4]

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[7] Experiments arranged as in Figs. 8 and 9 were checked in all cases by using a double string galvanometer and employing two contacts connected with one string on one side of the salt bridge and on the other side two contacts connected to the other string as in Fig. 10.

[8] Blinks, L. R., *J. Gen. Physiol.*, 1929–30, 13, 495.

SUMMARY

A negative variation in *Nitella* is unable to pass a spot killed by chloroform but can set up a negative variation beyond this spot when a salt bridge is put around it. It can likewise set up a negative variation in a cell of another plant if connected to it by two salt bridges.

[Reprinted from THE JOURNAL OF GENERAL PHYSIOLOGY, July 20, 1930, Vol. 13, No. 6, pp. 695–713]

THE KINETICS OF PENETRATION

II. THE PENETRATION OF CO_2 INTO VALONIA

BY A. G. JACQUES AND W. J. V. OSTERHOUT

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Accepted for publication, September 19, 1929)

In a previous paper[1] it was shown that in the cells of *Valonia macrophysa* the undissociated CO_2 very soon comes into equilibrium with that in the sea water outside. As this does not tell us in what form CO_2 penetrates[2] it seemed desirable to study the time curves of penetration, for it has been shown[3] that if they follow an equation of the first order the velocity constant, V_M, which is found when molecules alone enter, should theoretically behave like the velocity constant, V_A, found when the ion pair $H^+ + HCO_3^-$ alone enters: furthermore if both molecules and the ion pair $H^+ + HCO_3^-$ enter the velocity constant, V_{MA}, should act like V_M and V_A; but this is not true of V_{Na}, found when only the ion pair $Na^+ + HCO_3^-$ enters, or of V_{MNa}, found when molecules of HA enter in addition to the ion pair $Na^+ + HCO_3^-$. Similarly, if the internal pH value remains constant as the external pH value increases, V_M, V_A, and V_{MA} should, theoretically, remain constant, but V_{Na} and V_{MNa} should increase. If the internal pH value rises, V_M, V_A, and V_{MA} should fall off but V_{Na} should rise: and V_{MNa} should fall or rise, depending on whether molecules or ions penetrate more rapidly.

Furthermore, if we keep the concentration of undissociated CO_2

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[1] Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925–26, **9**, 255. This paper contains references to previous work on the penetration of weak acids into living cells. For experiments on the penetration of CO_2 into *Valonia* see Brooks, M. M., *Pub. Health Rep.*, 1923, **38**, 1470.

[2] *Cf.* Osterhout, W. J. V., Some aspects of cellular physiology, in Lectures on Plant Pathology and Physiology in Relation to Man, Mayo Foundation Lectures, Philadelphia, 1926–27, p. 179. Also *Proc. Soc. Exp. Biol. and Med.*, 1928–29, **26**, 192. A different conclusion was reached in a previous paper (1).

[3] Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 261.

constant while increasing that of HCO_3^- and $CO_3^{=}$ (by raising the pH value and concentration of total[4] CO_2 in the sea water) we should expect an increase in the rate in case HCO_3^- or $CO_3^{=}$ enters. It was therefore decided to compare the time curves of penetration of CO_2 at high and low pH values, *e.g.*, at pH 4.8 at which the CO_2 present in sea water is considered to be almost completely undissociated, and at pH 6.8 where the dissociation is over 75 per cent.[5]

The experiments were carried out on *Valonia macrophysa* in Bermuda at a temperature of 20° to 24°C.; the temperature did not vary more than 0.5°C. during any one run.

Samples of sea water were prepared in 2-liter Winchester bottles, uniform as to the sea water used, but differing as to content of CO_2: the pH value was either 4.8 or 6.8. All the samples for the series reported here were prepared at one time. They were protected from change in pH value or CO_2 content by being filled without gas space, and having the rubber stoppers wired down. The CO_2 was introduced by adding the required amount of $NaHCO_3$, and the pH value was adjusted by adding 0.1 M NaOH or HCl. The pH value was determined colorimetrically, using the indicators of Clark and Lubs,[6] and Kolthoff's buffers[7] (with allowance for salt error).

The experiments on penetration were carried out in wide-mouthed bottles of about 125 cc. volume. All the bottles required for one run[8] were filled at one time. For this purpose one of the Winchester bottles mentioned above was unstoppered, and a rubber stopper carrying a wide siphon tube was inserted. About 100 cc. of the sea water was withdrawn to wash out the tube, and its place was taken by the same amount of "Nujol" drawn into the Winchester bottle as the sea water ran out. The small bottles were then quickly filled, the end of the siphon tube being allowed to dip below the surface of the entering liquid in order to cut down the loss of gas. Each bottle was stoppered with a rubber stopper as soon as it was filled, without gas space above the liquid. A certain amount of gas escaped from the main volume of sea water as its level fell in the Winchester bottle, but the layer of "Nujol" on top served to reduce this loss.

It is desirable to have cells of uniform size and shape but as this was impossible we endeavored to have the same volume and surface exposed in each case. To get consistent results, the cells must be selected so that the same volume and surface are exposed in the determination of each point in a run, and the stirring must be uniform. To achieve the first condition we collected a large number of cells and divided them into four groups of the following approximate volumes, 0.75 to 0.6 cc., 0.5 to 0.4 cc., 0.4 to 0.3 cc., and 0.3 to 0.1 cc. For each small bottle we selected 1 cell from the first group and 2 cells from each of the other groups. And in each of these three groups we chose equal numbers of approximately spherical, and approximately ellipsoidal cells. The total volume of cells in each small bottle was thus about 2.7 cc. The second condition, uniform stirring, was secured by means of a special stirrer (Fig. 1). This consisted simply of a square brass shaft, *A* (Fig. 1), revolving on the bearings *D* and D_1, recessed on opposite sides. The six recesses, *B*, had curvatures which would fit the curvature of the type of bottle used throughout this work. Each recess was provided with a set of two brass rods, *C*, one on each side and so arranged that a bottle could be slipped easily between them (see the lower left recess of the figure). Sliding on each set of rods was a curved brass bar *E* ($\frac{3}{8}$ inch wide). This was fitted with a handle and it was pressed towards the shaft by means of steel springs *F* coiled around the rods. The bar *E* was prevented from approaching the shaft too closely by the brass pins, *G*, drawn through the rods. With no bottle in place the distance between the center of the bar and the center of the recess was about $\frac{1}{4}$ inch less than the diameter of a bottle. When it was necessary to insert a bottle the bar could be pulled out slightly by the handle and the bottle put in. On releasing the handle the bar held the bottle firmly against the shaft. It was possible to insert any bottle while the stirrer was in motion. Thus we could start the stirrer and without stopping it determine all the points required for one run. This contributed greatly to the uniformity of stirring throughout a run, and since the shaft was always revolved at the same rate (4 revolutions per minute) the stirring was quite uniform throughout the work. This was about the best rate since the bottles were turned over just fast enough to cause the cells to describe a figure of eight motion in the bottle.[9] This served not only to keep the

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[4] By total CO_2 is meant the undissociated and dissociated CO_2 taken together.

[5] *Cf.* 1, p. 259. Also Güntelberg, E., and Schiödt, E., *Z. physik. Chem.*, 1928, 135, 393.

[6] Clark, W. M., Determination of hydrogen ions, Baltimore, 3rd edition, 1928.

[7] Kolthoff, I. M., and Furman, N. H., Indicators, New York, 1926, pp. 147–48.

[8] By "run" is meant all the separate determinations required for the determination of one time curve at constant pH value and content of CO_2.

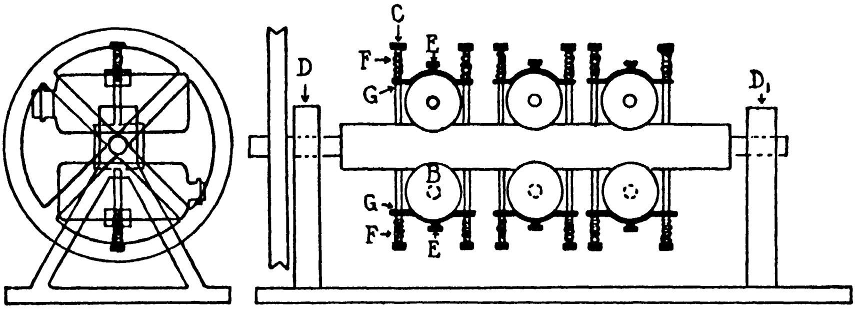

FIG. 1. Diagram of stirrer

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[9] That is, across the bottom, then ascending diagonally to the top, across the top, and descending diagonally to the bottom.

cells in motion, but also to keep the CO_2 content of the sea water uniform throughout.

In carrying out an experiment, *i.e.*, the determination of a single point on a time curve, the stopper of a bottle was sharply jerked out (using a file with a sharpened point as a lever), the cells were at once dropped in, the stopper was reinserted tightly without gas space, and the bottle was immediately placed on the stirrer. A small loss of gas was inevitable when the stopper was removed, since there was a momentary vacuum created over the liquid. By jerking the stopper out quickly, this loss was reduced. The whole operation, from the selection of the cells to the commencement of stirring, could be carried out in from 5 to 10 seconds; so that, except in the case of the 1-minute experiments, the proportion of non-uniform stirring was small. About 2 seconds before the end of the experiment the bottle was taken from the stirrer and the cells were poured out onto a nichrome screen over a beaker. As quickly as possible the sap was extracted for analysis.

For the collection of the sap, pipettes were prepared from 5 mm. thick wall glass tubing. This was drawn down to a point about 2.5 mm. in diameter with an opening of approximately 0.5 mm. The point was then ground off at 60° to a sharp but slightly jagged edge. Three pipettes were used, delivering 1.1930±0.0015 cc., 0.8830±0.0008 cc., and 0.8857 ±0.0010 cc. of distilled water, the last drop being blown out in each case. It was found that the sap always left a thin film of gelatinous material in the pipette, so that to attain the accuracy mentioned it was necessary to treat the pipette with chromic acid solution between fillings.

The extraction of the sap was accomplished by bringing the cell against the sharp edge of the pipette and imparting a twisting motion to it. The saw-like edge instantly cut through the tough cellulose wall, and the sap could be expressed into the pipette by gentle squeezing. As the cell became deflated the protoplasm was detached, and mixed with the sap. To avoid driving this into the pipette the last drop of sap was not extracted. After extraction of the necessary amount of sap, those cells which were not punctured were returned to ordinary sea water for observation of injury and kept under observation for several days:[10] when injury occurred the experiment was rejected.

In sampling, our routine was to proceed from the smallest cells up. An occasional cell softened during the experiment, and as we had no way of determining whether this indicated an increased permeability of the protoplasm we rejected all such cells. Sometimes on attempting to extract the sap from the smaller cells, the rupture spread beyond the rim of the pipette, and part or all of the sap was lost. For these reasons uniformity in the size of the cells taken for analysis was not attained.

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[10] The best criterion of injury is microscopic observation (under the high power) which should be frequently carried out during the experiment as reversible injury may occur (*i.e.*, injured spots may appear during the experiment which may become normal in appearance when the cell is returned to its normal environment).

Immediately after extraction the sap was transferred to a Van Slyke constant pressure carbon dioxide apparatus, and the analysis was carried out according to the method of Van Slyke.[11] In every case (regardless of whether the sample was slightly greater or less than 1 cc.) the total volume was made up to 2.5 cc. so that the tables given in the paper quoted could be used.

The accuracy of the analysis for CO_2 by the Van Slyke method can be calculated from the figures given by Van Slyke in the paper quoted above. The probable error of the mean is about 0.17 per cent of the mean for a concentration range comparable with that of our solutions. This error is assumed to include the error involved in measuring out the solution by pipette. The error produced by the loss of gas from the sap during transfer to the Van Slyke apparatus may be more serious than the formal error of analysis. But it is certainly small in comparison with the variation of the material. For this reason we did not adopt the method of transfer suggested by Van Slyke and Neill,[12] by which the sample is delivered directly into the gas burette. Instead we kept this error as low as possible by transferring the sap rapidly.

A further source of error was the variation of the CO_2 content of the sea water in a single run. This was unavoidable in our method of filling the small bottles. The possibility of carrying out this operation by causing the sea water to displace an oil like "Nujol" upwards, or mercury downwards, was rejected because the oil could not be displaced cleanly and the cells became coated with grease, while mercury was rejected because of the toxic nature of its salts. To evaluate this error we filled a number of bottles with CO_2-containing sea water at pH 4.8, in the usual manner, and then unstoppered them at intervals during 2 hours, and analyzed for CO_2. The probable deviation from the mean was found to be 0.007 cc. or 1.1 per cent.

Unless otherwise stated the curves are drawn free-hand to give a rough fit.

The data for four time curves at pH 4.8 and four at pH 6.8 are given[13] in Table I.

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[11] Van Slyke, D. D., *J. Biol. Chem.*, 1917, **30**, 347.

[12] Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, **61**, 523.

[13] It may be of interest to compare the figures for penetration of CO_2 into living *Valonia* with those obtained by Northrop (Northrop, J. H., *J. Gen. Physiol.*, 1928–29, **12**, 435, Table III) for penetration through a (previously well dried) collodion membrane separating a solution of NaOH from a solution of CO_2. Since in his experiments CO_2 was dissolved in distilled water we may use for comparison our results at pH 4.8. If we consider that 0.1 mg. CO_2 penetrates in 1 minute per cubic centimeter of sap at 20°C. when the external concentration is 0.904 mg. CO_2 per cubic centimeter sea water the pressure at the start is 0.5 atmosphere (allowing 0.009 mg. CO_2 per cubic centimeter sap at the start) so that

If the penetration is a simple diffusion we might expect the time curve to obey an equation of the first order. The velocity constants calculated from the equation,[14]

$$K = \frac{1}{t} \log \frac{a}{a - x}$$

are shown in Fig. 2. Since they fall off from the start it might seem that the equation for a dimolecular reaction might give better values: accordingly calculations were made with the dimolecular equation

$$K_1 = \frac{1}{t(a - b)} \log \frac{(a - x)b}{(b - x)a}$$

putting $a = 1.00$ and $b = 1.35$. Since this gives a more constant value of K_1 (as shown in Fig. 2) it might seem that the dimolecular equation should be employed, and that possibly a reaction occurs between the penetrating substance and some constituent of the protoplasm. We find, however, that when we multiply all the ordinates of a time curve by the same factor (varying the factor from curve to curve) so as to make the equilibrium value the same in all cases, the curves agree within the limits of experimental error (Fig. 3). Hence the times for half completion must be approximately the same in all cases. This would not be the case if they obeyed a dimolecular equation[15] (unless

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at 1 atmosphere 0.2 mg. CO_2 would penetrate per minute per cubic centimeter of sap and for a cell containing 0.41 cc. the amount penetrating would be 0.2 (0.41) = 0.082 mg. Such a cell may be regarded as having a surface of 2.8 sq. cm. If the surface were 1 sq. cm. the amount penetrating per minute would be 0.082 ÷ 2.8 = 0.0292 mg. and the amount penetrating per day would be 42 mg. = 21.2 cc. The figure given by Northrop is 8.6×10^{-4} cc. per square centimeter per day for a membrane 1 cm. thick. If we consider the protoplasm to be in the neighborhood of 0.001 cm. thick this would give 0.86 cc. per day for collodion but as the non-aqueous part of the protoplasm is undoubtedly much thinner this figure is probably much too small. It would seem therefore that penetration may be of the same order of magnitude for such collodion membranes and for *Valonia.*

[14] Common logarithms are used for convenience and a is put equal to the final equilibrium value of the time curve: this value corresponds approximately to that of the undissociated CO_2 in the sea water but is not equal owing to differences in solubility. (*Cf.* 1.)

[15] Another way of looking at the matter is to say that if the curves agree when multiplied up (so as to have the same equilibrium value) the time for half com-

TABLE I

Increase of Total CO_2 (Observed Value Less the Amount Present at the Start) in Cells Unlike in Size and Shape. Each Figure Represents the Average of 20 or More Cells

Time	pH of sea water 6.8				pH of sea water 4.8			
	mg. of total CO_2 per cc. of sap				mg. of total CO_2 per cc. of sap			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 1	Exp. 2	Exp. 3	Exp. 4
min.								
1	0.035	0.044	0.044	0.062	0.100	0.149	0.071	0.054
3	0.094	0.134	0.095	0.115	0.210	0.243	0.200	0.180
5	0.147	0.225	0.146	0.143	0.315	0.404	0.231	0.271
7	0.163	0.254	0.204		0.494	0.560	0.304	0.317
9	0.186	0.317	0.217		0.541	0.583	0.359	0.328
12	0.224	0.397	0.240	0.266	0.603	0.696	0.403	0.441
15	0.242	0.433	0.258	0.302	0.651	0.766	0.480	0.533
20	0.269	0.527	0.302	0.307	0.719	0.874	0.545	0.631
30	0.302	0.569	0.391	0.378	0.833	0.969	0.645	0.748
45	0.353	0.601	0.439	0.408	0.862	1.016	0.712	0.811
60	0.396*	0.649*	0.452	0.440	0.954	1.116*	0.791	0.891
120	0.395	0.643	0.482	0.456	0.990	1.049	0.791	0.891

* This is taken as the equilibrium value.

pletion must be the same for all as is the case when they are of the first order and all have the same velocity constant. But if they are of the second order this is not true. For example, if the two reactants are equal at the start and if the velocity constant is 1 we have as the time for half completion, t, the following:

$$t = \frac{x}{a(a - x)}$$

where a is the original concentration of each reactant or the final equilibrium value of x (x being the amount of substance formed by the reaction). Since $x = 0.5\ a$ at half completion, we have

$$t = \frac{0.5a}{a^2 - 0.5a^2} = \frac{1}{a}$$

that is, t is inversely proportional to a. Hence if with different concentrations of reactants the curves have the same velocity constant, it follows that the times for half completion cannot be the same and the curves will not be identical when the equilibrium values are made equal by multiplying the ordinates.

If the reaction is dimolecular we should expect the velocity constant to increase

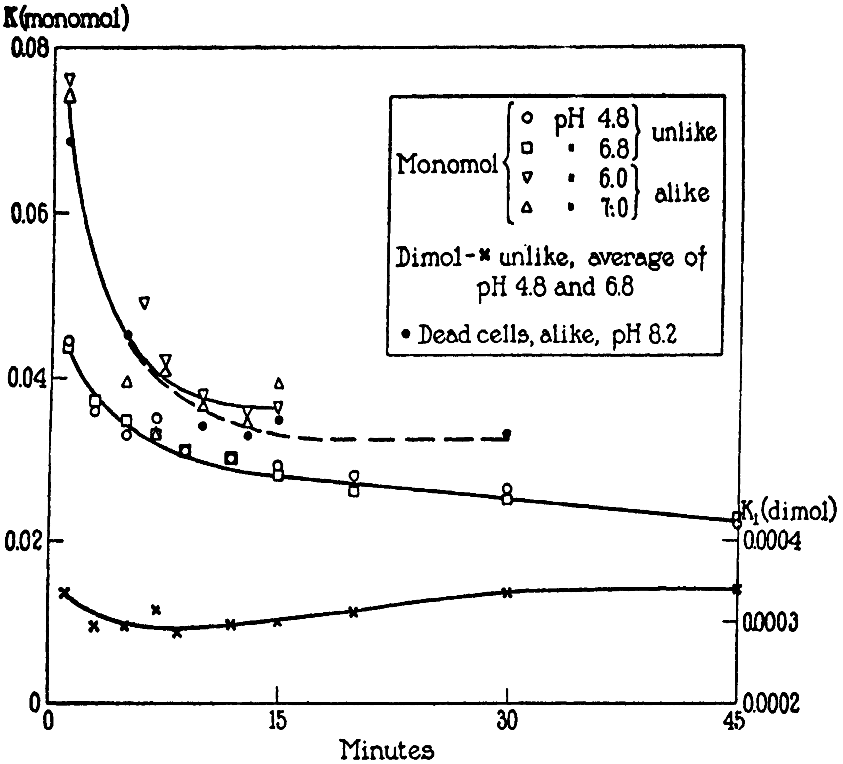

FIG. 2. Monomolecular velocity constants for living and dead cells calculated from the equation $K = \frac{1}{t} \log \frac{a}{a - x}$, where x is the increase of total CO_2 in the sap and $a = 1.00$, the equilibrium value: also dimolecular constants for living cells calculated from the equation $K_2 = \frac{1}{t(a - b)} \log \frac{(a - x)b}{(b - x)a}$, where $a = 1.00$, $b = 1.35$.

as the external concentration of CO_2 increases, but Fig. 3 shows that this is not the case (for example, when we compare experiments at the same external pH value).

If a compound is formed between the protoplasm and CO_2, we should expect that in the case of (H^+, HCO_3^-) ion pairs we should have to deal with two consecutive dimolecular reactions: $H^+ + HCO_3^- \rightarrow H_2CO_3$ at the surface, followed by H_2CO_3 + protoplasm $\rightarrow$ H_2CO_3 protoplasm, and even a termolecular reaction: $H^+ + HCO_3^-$ + protoplasm $\rightarrow$ H_2CO_3 protoplasm. In either case the imposition of the second reaction on the reaction where molecules alone are involved ought to alter the form of the curve. The fact that it does not might be regarded as evidence for the view that (H^+, HCO_3^-) ion pairs do not penetrate. And as we pointed out above there is also evidence for the view that (Na^+, HCO_3^-) ion pairs do not penetrate.

their velocity constants happened to fall in just the right relation to the concentrations employed: this would require an extraordinary set of coincidences) but would if they followed the monomolecular formula. The question arises, How can they follow this formula when the velocity constants fall off as in Fig. 2? Now in the case of dead cells,[16] where there would seem to be no question that the ordinary

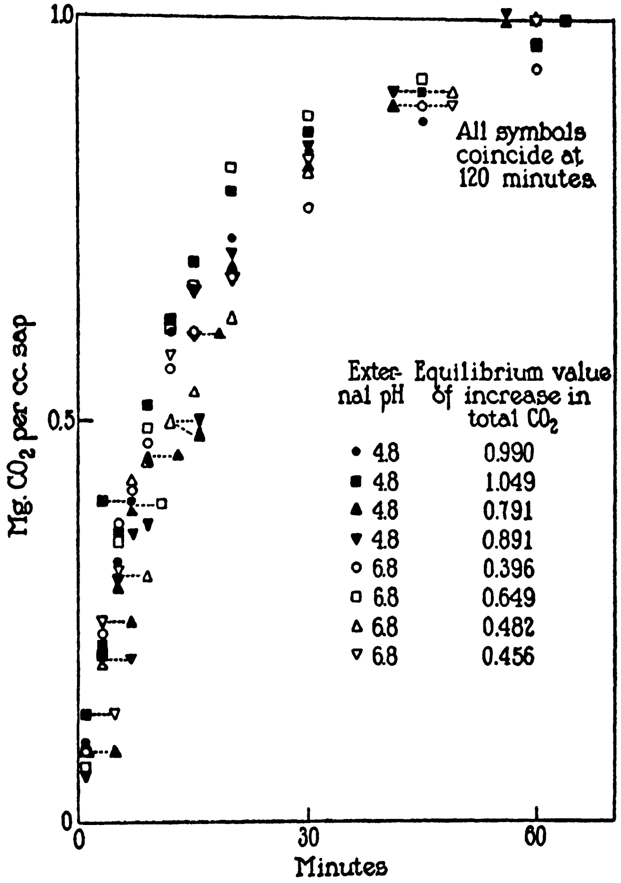

FIG. 3. Shows the result of bringing all the curves (for unlike cells) to the same final value by multiplying all the ordinates of a curve by the same factor (using a different factor for each curve).

To avoid crowding some symbols have been displaced to one side but these are connected by a line to the point where they belong.

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[16] These often show a slightly higher velocity constant at pH 4.8 than at pH 6.8 which is to be expected since the diffusion constant of CO_2 is greater than that

diffusion formula is followed, the constants often fall off in much the same way. It would therefore seem possible to regard the curves as of the first order but having a "velocity constant" which falls off from the first as would be the case, for example, if the temperature were falling. They can be fitted more or less closely[17] by means of the empirical formulae[18]

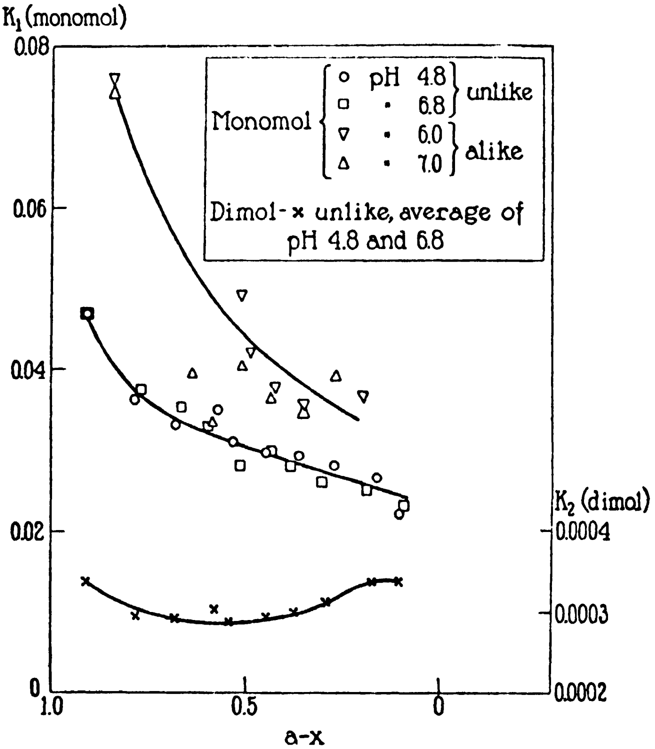

FIG. 4. As in Fig. 3, but with $a - x$ as abscissae

$$K_2 = \frac{ba}{tx} \ln_e \frac{a}{a-x} - x \text{ and } K_2 = \frac{bx^n}{t} \ln_e \frac{a}{a-x}$$

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of Na_2CO_3 (*cf.* Internat. Critical Tables, V, 63, 67) and the cell wall is so permeable that only ordinary diffusion would appear to be involved. We might apply here the equations employed elsewhere[3] but this is not necessary since no protoplasm is present and consequently there is only one phase (the cell wall being so permeable that it need not be regarded as a distinct phase).

[17] These may fit the latter part of the curve better than the earlier part.

[18] In these formulae K_2 is not independent of a.

other empirical formulae may be used in similar cases, *e.g.*,

$$K_2 = \frac{1}{t} \ln_e \frac{a}{a - bx^n} \text{ and } K_2 = \frac{1}{t} \frac{ab}{x^n} \ln_e \frac{a}{a - x}$$

Let us now consider the behavior of the "constants" more closely. When they are plotted against $a - x$ (as in Fig. 4) the curve does not differ greatly from that with time as abscissae (Fig. 2). It is better to plot the constants calculated for short time intervals at various points along the curve: these may be called "momentary constants" for convenience. They are calculated from the smoothed curve (*A*, Fig. 5) obtained by averaging all the points shown in Fig. 3. For this purpose we employ the formula

TABLE II

Increase of Total CO_2 (Observed Value Less the Amount Present at the Start) in Cells Alike in Size and Shape. Each Figure Represents the Average of 20 or More Cells

Time in minutes		1	5	6	7	7.5	10	13	15	30	120
Live cells	pH of sea water = 6	0.159		0.489		0.510	0.573	0.649	0.709		0.990
	pH of sea water = 7	0.073	0.171		0.193	0.236	0.266	0.305	0.337		0.469
Dead cells	pH of sea water = 8.2	0.181	0.501				0.668	0.777	0.844	1.112	1.240

$$K_m = \frac{1}{t_2 - t_1} \log \frac{a - x_1}{a - x_2}$$

where x_1 is the total CO_2 penetrating in the time t_1 and x_2 is that penetrating in the time[19] t_2. The results of these calculations, shown in Figs. 6 and 7, offer nothing essentially new.

It is therefore clear that the constants really fall off and it might be natural to ascribe this to differences in size and shape as the result of which some cells would have a relatively greater surface and consequently reach equilibrium sooner than the others so that when the whole group is considered penetration appears relatively rapid at the start.

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[19] In the first part of the curve the interval between t_1 and t_2 is in seconds but in the latter longer intervals are used.

To test this suggestion would require a quantity of cells uniform in size and shape which there seemed to be no immediate prospect of obtaining. Eventually, however, a supply was secured and the experiments were carried out. The results obtained are shown in Table II and Fig. 5; the velocity "constants" fall off as is shown in Figs. 2, 4, 6, and 7.

The experiments were carried out as before except that a Van Slyke constant volume apparatus was used and in order to avoid loss of CO_2 higher pH values (6 and 7) were used. The temperature varied between 18° and 20°C.

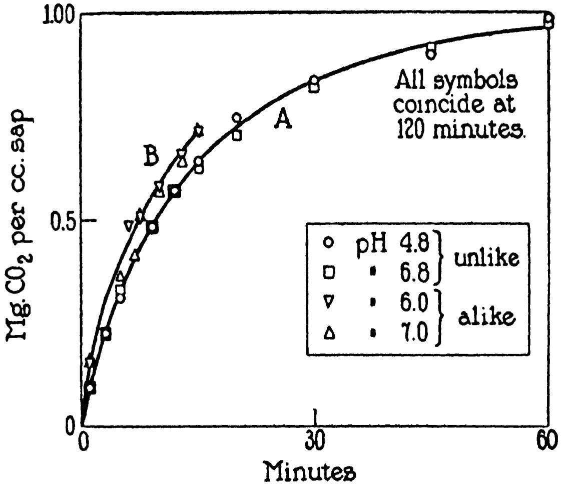

FIG. 5. Time curves showing increase of total CO_2 in the sap of living cells, (*A*) unlike in size and shape, (*B*) similar in size and shape. The ordinates have been multiplied to make the equilibrium value 1.0 in each case.

It is evident that if some of the cells were more permeable than others the effect would be the same as if their surface were relatively large and this would cause a falling off of the "constants." We are inclined to accept this explanation.

Experiments on dead cells bear out this idea and are in harmony with investigations on the penetration of KIO_3 made several years ago by Mr. W. C. Cooper, Jr. (in collaboration with the senior author). Studying the penetration of KIO_3 into dead cells of the same size and shape it was found that the velocity "constants" sometimes fell off and sometimes remained practically constant. The latter condition was favored by using cells whose permeability was similar because they were freshly killed. When such cells were mixed with others that had stood for a long time in sea water after killing (so that their walls were partially disintegrated) the constants fell off much more markedly. This was doubtless due to the fact that some of the dead cells were more permeable than the others.

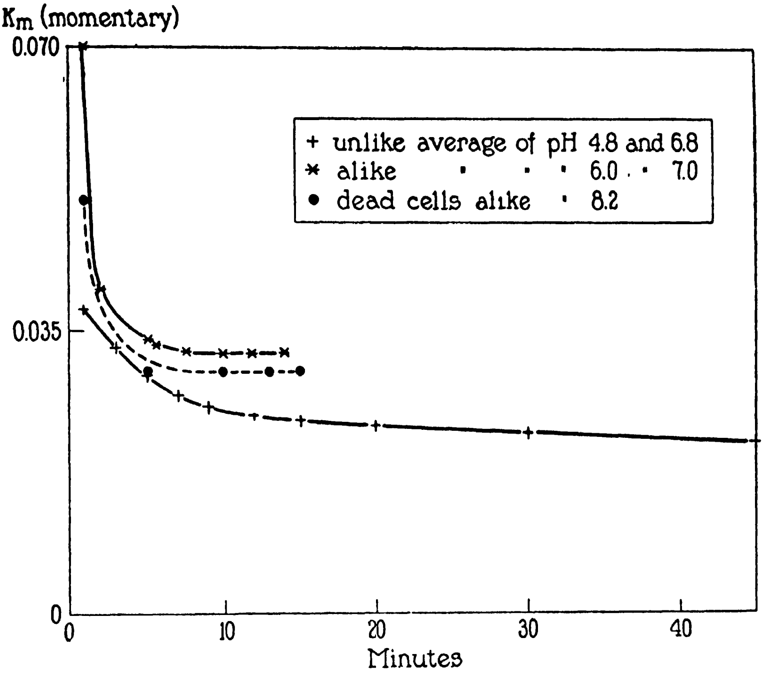

FIG. 6. "Momentary" constants calculated for short intervals along the time curves from the formula $K_m = \frac{1}{t_2 - t_1} \log \frac{a - x_1}{a - x_2}$, where $t_2 - t_1$ represents a very short time interval, x_1 is the increase in total CO_2 in the sap at the time t_1, x_2 the increase at the time t_2, and a the equilibrium value.

Since under the most favorable conditions the velocity constants do not fall off when dead cells of the same size and shape are employed, we are inclined to attribute the falling off with cells of the same size and shape, whether alive or dead (for dead cells[20] see Table II and

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[20] These experiments were carried out with sea water at pH 8.2.

Figs. 2, 6, and 7), to differences in permeability which cause a more rapid penetration on the part of certain cells: for when these have reached equilibrium penetration continues in the others so that the process for the whole group seems relatively rapid at the start.[21]

There is always a small amount of CO_2 present in the sap even before its exposure to the CO_2-bearing sea water, which would tend to make the velocity constant too high at the start. We have determined this amount a number of times on freshly gathered cells which may be assumed to be growing, and on cells which have been kept in the laboratory and have practically ceased to grow. Very little difference was found between the two groups and the average amount was 0.009 mg. of CO_2 per cubic centimeter of sap. This was subtracted from the observed figures before calculating the velocity constants. It may

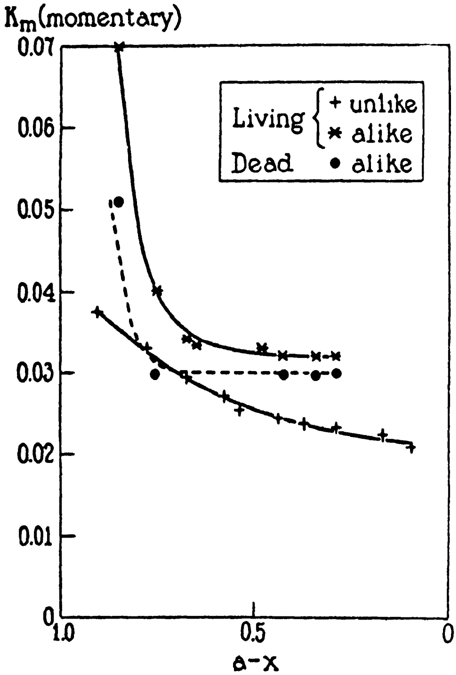

FIG. 7. As in Fig. 6, but with $a - x$ as abscissae

be added that the production of CO_2 during the experiment is too small to affect the result.

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[21] It might be thought that if there is much difference in permeability an occasional sample would consist largely of cells of high or of low permeability. The experiments with cells of the same size and shape are not extensive enough to settle this point: the figures for one set of determinations are given in Table III.

Another factor which might affect the velocity of penetration is the change in the pH of the sap as the CO_2 concentration increases. We have investigated this change experimentally. Fig. 8 gives our results. Our experiments were not exhaustive but it seems safe to say that the rate of change in pH of the sap is dependent chiefly on the rate of accumulation of CO_2 in the sap, and that it is independent of the pH of the sea water, except as a lower pH of the sea water means that a greater proportion of CO_2 is in molecular form. Assuming that when the pH of the sap has fallen to 4.7 the CO_2 is present almost entirely as molecules, we see that this condition of association may be reached early in the runs (and might be reached earlier in the protoplasm than in the sap). We are inclined to think that this means that there

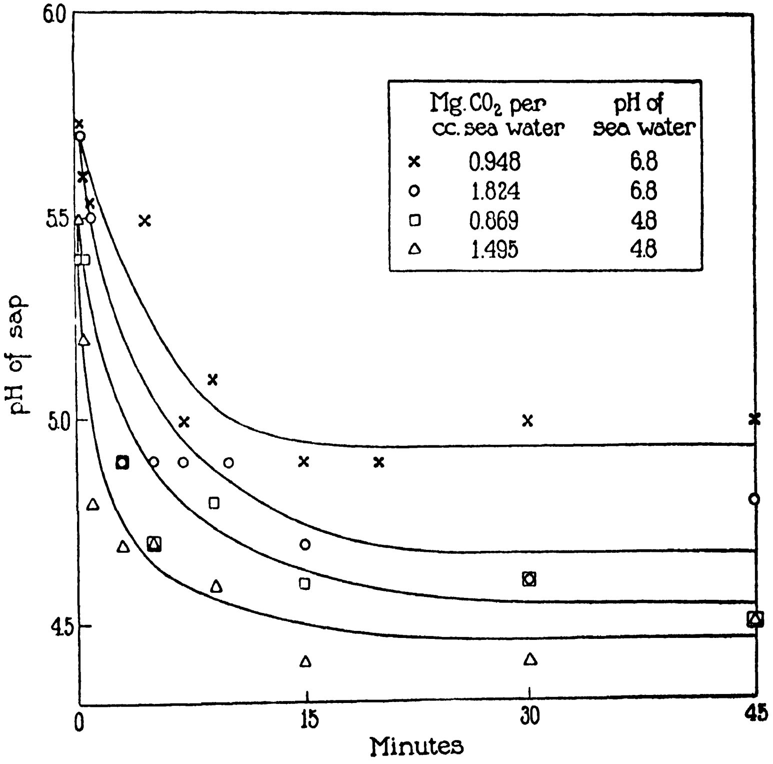

FIG. 8. Changes in the pH value of the sap during the entrance of CO_2

is little or no penetration of the ion pair $Na^+ + HCO^-$ for the entrance of $NaHCO_3$ would raise the pH, opposing the drop produced by the entrance of molecules of CO_2.

If it is chiefly the molecules which enter, the decrease of internal pH value during penetration would tend to make the velocity constant appear greater[3] and greater as long as it continued, but as a matter of fact, the value of K is found to fall off at the start and it continues to fall off after the internal pH value has presumably become constant.[22]

In case the outside volume is small compared to that of the cells the external concentration will decrease as time goes on, but this should not cause a falling off in the velocity constant since the calculation is referred to the equilibrium value of CO_2.[23]

The essential point is not the constancy of K but its behavior at different pH values. If we regard the time curves as belonging to the first order (but with K falling off from the start) we may conclude that the velocity constant is practically the same at high and low pH values. For reasons pointed out elsewhere[3] this would be expected if the chief substance penetrating were undissociated CO_2 or the ion pair $H^+ + HCO_3$, but not if $NaHCO_3$ entered to any noticeable extent.

The question whether the time curve is of the first order need not be raised if we consider only the initial rate $\left(\frac{dS_i}{dt}\right)_b$ (*i.e.*, the rate when t is very small) for then all time curves approximate the zero order (*i.e.*, a straight line) since the outside concentration remains approximately constant and practically none of the penetrating substance moves outward. It has been shown elsewhere[3] that when both undissociated molecules and the ion pairs $H^+ + HCO_3^-$, $Na^+ + HCO_3^-$ and $K^+ + HCO_3^-$ enter we may write

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[22] As the pH value of the sap decreases the per cent of loss during manipulation increases because more of the CO_2 is in volatile form but this would not cause a falling off in the velocity constant because it affects the equilibrium value even more than the early values.

[23] The case is similar to that of a reversible monomolecular reaction $A \rightleftharpoons B$ where A decreases but the time curve of increase of B is monomolecular (*cf.* Mellor, J. W., Higher mathematics for students of chemistry and physics, London 1922, p. 228) when the calculation is based on the equilibrium value.

In these experiments the volume of cells was relatively small in comparison with that of the sea water and in no case did the external concentration of total CO_2 fall off more than 2 per cent during an experiment.

$$\left(\frac{dS_i}{dt}\right)_b = P_M M_o + P_A H_o A_o + P_{NaA} Na_o A_o + P_{KA} K_o A_o$$

where M_o is the molar concentration of undissociated CO_2 outside (including H_2CO_3), H_o the external concentration of H^+, Na_o that of Na^+, K_o that of K^+, and A_o that of HCO_3^-; S_i is the total CO_2 inside, P_M is the amount of M_o entering in unit time through unit surface under unit pressure, P_A is the corresponding value for the ion pair $H^+ + HCO_3^-$, P_{NaA} that for the ion pair $Na^+ + HCO_3^-$, and P_{KA} that for the ion pair $K^+ + A^-$.

The results here given (together with unpublished figures) show that when the equilibrium values are made the same by multiplying the ordinates the time curves agree closely. For example, when all the curves are brought (by multiplication of the ordinates) to the equilibrium value 1.00, the average for the first minute is 0.0965 for pH 4.8 and 0.0960 for pH 6.8. With uniform cells (which furnish the most decisive test) the values for the increase at pH 6 at the end of the first minute are 0.1568, 0.1582, and 0.1605, giving an average of 0.1585. In order to bring the equilibrium value (0.990) to the standard (1.00) we must multiply it by 1.01 and we accordingly multiply 0.1585 by the same factor giving 0.160. How does this compare with the result at pH 7? The values at the end of the first minute are 0.0728, 0.0724, 0.0738, giving an average of 0.073. Since the equilibrium value 0.469 must be multiplied by 2.132 to bring it to the standard (1.00) we multiply 0.073 by the same factor, giving 0.156 which is very close to the value at pH 6 (0.160).

It would seem that the value of $\left(\frac{dS_i}{dt}\right)_b$ is practically the same at low and at high pH values. As the equilibrium values are the same the values of M_o must also be the same (provided the internal pH values are approximately the same, as seems to be the case). Now at pH 4.8 the concentration of HCO_3^- approaches zero so that the value of $P_A H_o A_o + P_{NaA} Na_o A_o + P_{KA} K_o A_o$ reduces almost to zero and when $\left(\frac{dS_i}{dt}\right)_b$ and M_o have the same values at pH 6.8 and at 4.8 the value of the expression $P_A H_o A_o + P_{NaA} Na_o A_o + P_{KA} K_o A_o$ must be nearly zero at pH 6.8 even though the concentration of HCO_3 and of A_o is relatively large. This can only mean that there is little penetration of the ion pairs $Na^+ + HCO_3^-$ and $K^+ + HCO_3^-$.

In other words, the results indicate that adding $NaHCO_3$ to the external solution has little effect on the rate of penetration provided we do not at the same time change the external concentration of undissociated CO_2 so that we conclude that there is very little penetration of HCO_3^- or $CO_3^=$.

It may be objected that since the value of $\left(\frac{dS_i}{dt}\right)_b$ is not directly determined there may be some uncertainty in this procedure. We have tested this matter experimentally in the following way.

TABLE III

Increase of Total CO_2 (Observed Value Less the Amount Present at the Start) in Cells Alike in Size and Shape at the End of 2 Minutes. Each Figure Represents the Average of 10 or More Cells

pH of sea water	mg. of total CO_2 per cc. of sap						
	Individual experiments						Average
6	0.0111	0.0161	0.0070	0.0130	0.0305	0.0073	0.0142
7	0.0130	0.0146	0.0071	0.0124	0.0297	0.0067	0.0139

Differences between the columns are due to differences in the concentration of CO_2 in the sea water in the different experiments but in each column the concentration of undissociated CO_2 in the sea water was the same for both pH values.

Two samples of sea water, one at pH 6 and the other at pH 7, containing approximately the same amount of free CO_2 were prepared and placed in 125-cc. bottles. Then by means of an apparatus similar to that used by Osterhout and Dorcas[1] gas was circulated between the two solutions for 2 hours, at the end of which time it was assumed that the CO_2 tension was the same in each. The two solutions were then used in penetration experiments on cells of about the same size and shape. The time of exposure was 2 minutes. The results of six pairs of determinations are given in Table III.

It will be seen that the amount of CO_2 penetrating during the 2 minutes of exposure in these experiments is much smaller than that penetrating in the same time in the experiments reported above. The reason for this is that the smaller amounts of CO_2 can be distributed equally between the two samples of sea water in a reasonable time, while at the same time the losses due to diffusion through the rubber part of the distributing system, and during the penetration experiments are very much cut down.

In spite of the small amount of CO_2, by reducing the gas to the smallest volume permitted by the Van Slyke constant volume apparatus, we have measured it with an error which does not exceed twice the error of the previous measurements.

Table III shows that the average values of 6 pairs of experiments check within 2 per cent which is well within the experimental error which we believe to be not greater than 6 per cent. It would therefore seem that the rate of penetration depends only on the concentration of undissociated CO_2 and not on that of HCO_3^- or $CO_3^=$ since in these experiments the concentration of ions could be greatly varied without affecting the rate of penetration as long as the concentration of undissociated CO_2 remained unaltered.

SUMMARY

The rate of penetration of CO_2 into living cells of *Valonia* has been studied at high and low pH values.

The time curve of penetration appears to be of the first order but with a "velocity constant" which falls off from the start.

The evidence indicates little penetration of ions. This is shown by (*a*) the similarity of velocity "constants" at high and low pH values, (*b*) the rate of penetration, which remains constant as long as the external concentration of undissociated CO_2 remains constant no matter how much the concentration of ions varies.

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CALCULATIONS OF BIOELECTRIC POTENTIALS

I. EFFECTS OF KCl AND NaCl ON NITELLA

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In this paper the chemical and concentration effects of KCl and NaCl in *Nitella* (Table I) are examined with a view to mathematical treatment.

TABLE I

Chemical and Concentration Effects in Nitella

0.001 M KCl positive* to 0.01 M KCl	54.7 mv.**
0.001 M NaCl positive to 0.01 M NaCl	20.9 mv.***
0.01 M NaCl positive to 0.01 M KCl	82.9 mv. †
0.01 M NaCl positive to 0.005 M KCl + 0.005 M NaCl	68.8 mv. ‡

* *I.e.*, positive in the external circuit.

** Average of 25 experiments: probable error of the mean 1.6 % of the mean.

*** " " 23 " " " " " " 4.5 % " " "

† " " 8 " " " " " " 3.2 % " " "

‡ " " 16 " " " " " " 3.8 % " " "

The experiments were performed on *Nitella flexilis* at a temperature of 19° to 20°C. according to the technique previously described.[1]

The concentration effects were measured in the best range (0.01 M to 0.001 M[1]) on intact cells, giving values higher than those previously obtained[1] for KCl with intact cells (and more nearly like those obtained with cells having one spot killed with chloroform[2]); this is probably due to the fact that in the present experiments broader contacts were employed, reducing the protoplasmic resistance, which, as will be explained presently, gives a more accurate measure of the P.D. In addition broader air gaps were used, thus giving better values. Also the intact cells appear to have been in better condition than in the previous experiment (it is well known that injury causes the values to fall off). During the earlier experiment the criteria of injury and the methods of preparing the cells were not nearly so good as now. Furthermore, different concentrations of KCl were placed in succession on the same spot but in the previous experiment they were applied simultaneously to different spots.

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[1] Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1928–29, **12**, 761; *Proc. Soc. Exp. Biol. and Med.*, 1928–29, **26**, 838.

[2] When one spot is killed with chloroform the resistance which is called R_3 in the subsequent discussion is reduced: in the present experiments R_3 was reduced by broad contacts (without applying chloroform).

I wish to thank Mr. Philip R. Averell for the care which he has bestowed upon these experiments.

Following the conceptions developed in previous papers we may picture the protoplasm as shown in Fig. 1. The potential at the outer surface of X may be called E_{AX}, the corresponding value at C being

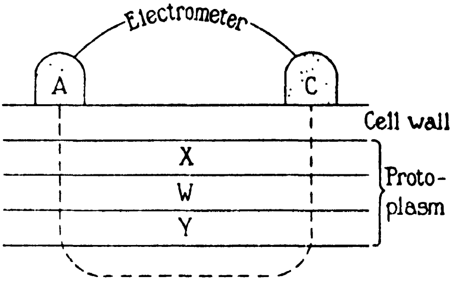

FIG. 1. Hypothetical diagram of the protoplasm of *Nitella*. The principal circuit is shown by the broken line.

E_{CX}. The sum of the other potentials at A may be called E_A, the corresponding value at C being called E_C. The total E.M.F. (which we may call E) is $E = E_A + E_{AX} + E_C + E_{CX}$. Since ordinarily E_A and E_C are equal and opposite they cancel out and we may put $E = E_{AX} + E_{CX}$.

The observed P.D. may be due to potentials of various kinds. The Donnan potential will be left out of account because the cell cannot very well be in equilibrium with two different solutions at the same time, and it does not seem probable that a local pseudoequilibrium can be set up in a few seconds at A or C between the aqueous layer,[3] W, of the protoplasm and the external solution (the case being entirely different from establishing a pseudoequilibrium between the outer surface of X and the external solution as discussed later on). If the system is on the way to a Donnan potential the P.D. will depend largely on mobilities. We shall therefore consider only diffusion potentials and phase boundary potentials. Let us begin with calculations on the basis of diffusion potentials alone.

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[3] To set up a new equilibrium in W would involve a new equilibrium between W and the sap.

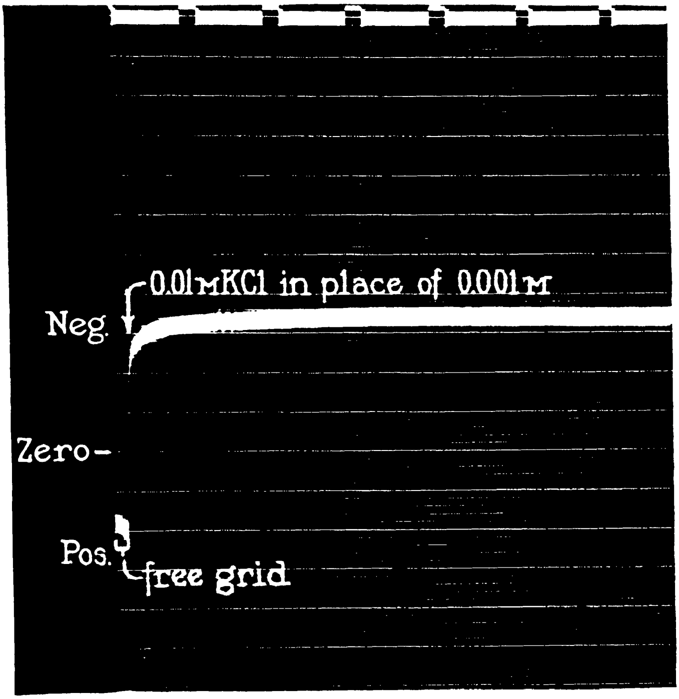
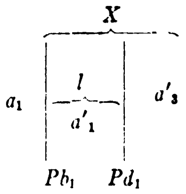


FIG. 2. Photographic record showing the effect of substituting 0.01 M KCl for 0.001 M KCl at A (Fig. 1) when C is in contact with 0.001 M KCl. Before the application of 0.01 M KCl at A the recording instrument is connected to C and to a cup containing 0.01 M KCl but the cell does not touch this cup (this part of the record is marked "free grid"). The left-hand end of the cell (A), which is imbibed with 0.001 M KCl, is wiped off and is then dropped into the cup and at the moment of contact the curve begins to rise: equilibrium is quickly established. The time marks represent 5-second intervals.

Calculation of Diffusion Potentials

If we apply 0.001 M KCl at A we may suppose that its behavior at the outer surface of X is like that with non-aqueous substances in general: it has been found that the P.D. set up with such substances reaches its full value very quickly and this seems to be also the case with protoplasm as shown in Fig. 2. The usual assumption is that the outer layer of the non-aqueous substance (which may be only a few molecules thick and which may be called l) very quickly comes into pseudoequilibrium with the aqueous solution. If this be the case and there is little or no phase boundary potential the mean ionic activity ($a_1 = \sqrt{a_{K}a_{Cl}}$) of KCl in the aqueous solution must be equal to that in X which is $a'_1 = \sqrt{a'_{K}a'_{Cl}}$. We then have the system

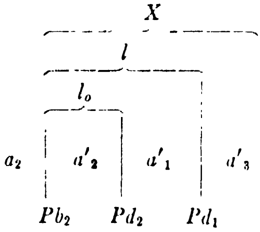


where a'_3 represents the mean ionic activities of all the salts in X where it is in equilibrium with tap water (in which the cell has been kept previous to the application of KCl), Pb_1 is the phase boundary potential and Pd_1 is the diffusion potential. The resulting P.D. in the outer portion of X at A may be called $E_{AX\,0.001}$ and that at C may be called $E_{CX\,0.001}$.

We may then write

$$E_{AX\,0.001} = E_{CX\,0.001} = Pb_1 + Pd_1$$

If we allow the solution of KCl 0.001 M to act for a few minutes, until the layer l has reached appreciable thickness, and then apply KCl 0.01 M we may conclude from the photographic records (which resemble Fig. 2) that in a very few seconds the outer part of l, which we may call l_o (consisting of only two or three layers of molecules), comes into pseudoequilibrium with the external solution so that we have the system



where a_2 is the mean ionic activity of KCl in the aqueous solution, a'_2 its mean ionic activity in l_o, and Pb_2 the phase boundary potential. The resulting P.D. may be called $E_{AX\,0.01-0.001}$ so that we may write

$$E_{AX\,0.01\text{-}0.001} = Pb_2 + Pd_2 + Pd_1$$

In order to obtain this we first measure the P.D. with 0.001 M KCl at both A and C (this is ordinarily not far from zero, otherwise the experiment is rejected). We leave these solutions in place for several minutes[4] and then replace the 0.001 M KCl at A by 0.01 M KCl and measure the P.D. immediately[5] thus obtaining the value of $E_{AX\,0.01-0.001} - E_{AX\,0.001}$ (concentration effect) which may be called $E_{\text{conc.}}$ so that we may write

$$\begin{aligned} E_{\text{conc.}} &= E_{AX\,0.01\text{-}0.001} - E_{AX\,0.001} \\ &= Pb_2 + Pd_2 + Pd_1 - (Pb_1 + Pd_1) \\ &= Pb_2 - Pb_1 + Pd_2 \end{aligned}$$

If the phase boundary potentials Pb_1 and Pb_2 are approximately equal we may put

$$E_{\text{conc.}} = Pd_2$$

It seems probable that electrical conditions in the protoplasm may correspond to some extent with the diagram in Fig. 3 (the circuits are, of course, not insulated from each other in the protoplasm). Here I_1 represents the current in the cell wall, I_2 that in X, I_3 that in Y and sap. E_1 is a small diffusion potential in a "horizontal" direction in the cell wall due to concentration effect (this is absent when we deal with chemical effects): E_2 is the diffusion potential in a "horizontal" direction in X and E_3 the potential in a "vertical" direction in X (the terms "horizontal" and "vertical" presuppose that the cell is oriented as in Fig. 1), R_1 is the "horizontal" resistance of the cell wall, R_2 the "horizontal" resistance of X, and R_3 the "vertical" resistance of X and Y at A and C plus the "horizontal" resistance of the sap.

Assuming that E_3 corresponds to what we have called $E_{\text{conc.}}$ above it is evident that we approach its true value when we make R_1 large and R_3 small. We make R_1 large by increasing the distance between the contacts (this was 2 to 5 cm.) and diminish R_3 by making the contacts broad (in this case 2 cm.): R_3 is often reduced by action currents (due to handling or to applications of solutions at a distance from the contacts) which have been shown by Blinks[6] to reduce the protoplasmic resistance. When the wall is imbibed with tap water as in most of these experiments the values, according to Blinks,[6] are approximately as follows: R_1 = 10 to 25 megohms probably R_2 = 100 megohms or more, R_3 = 0.4 to 0.6 megohms. We assume that E_1 corresponds to the concentration effect due to the cell wall[1] which acts very much like additional resistance in R_1 so that the greater E_1 (provided it does not exceed E_3) the nearer we come to[7] the true value of E_3 (for 0.01 *vs.* 0.001 M KCl the value of E_1 is about 17 mv.). Under these circumstances we measure practically the full value of E_3: this is apparent on setting up the usual Kirchoff equations (P.D. $= E_1 + I_1R_1 = E_2 - I_2R_2 = E_3 - I_3R_3$ and $I_1 = I_2 + I_3$) and is indicated experimentally by the near approach of the observed concentration effect of 0.01 *vs.* 0.001 M KCl (54.7 mv.) to the theoretical maximum (56.215 allowing for activities).

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[4] The P.D.'s usually remain quite constant during this interval.

[5] The measurements are made from photographic records similar to that shown in Fig. 2.

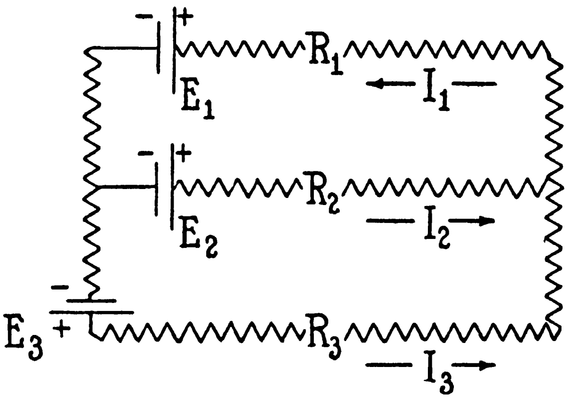

FIG. 3. Hypothetical diagram of electrical conditions in *Nitella*. See text.

The most reliable measurements are obtained when the cells are in the best condition and fairly free from action currents, since the latter often produce an effect analogous to that of an outward movement of K^+ from the sap[6] to the outside of the protoplasm. It is evident that if such a movement should occur when measuring the P.D. of 0.001 M KCl *vs.* 0.01 M KCl the effect would be to increase the concentration of 0.001 M KCl relatively more than that of 0.01 M KCl and hence to lessen the observed concentration effect.

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[6] Blinks, L. R., *J. Gen. Physiol.*, 1929–30, 13, 495. These values are for cells where the value of R_3 is reduced by broad contacts (as in the present case) or by frequent action currents.

The fact that the concentration effect of NaCl is so low must mean that the mobility of Cl^- in X is greater than zero.

[7] What is said of E_1 also applies to a certain extent to E_2.

Hence those measurements are chosen which run high for an entire lot of cells but the average of such a lot is taken rather than the highest individual value since the latter might be due to an E.M.F. in some layer other than X.

We may calculate the relative[8] mobilities by the usual equation (for 19°C.)

$$\text{P.D.} = 0.058 \frac{u - v}{u + v} \log \frac{a'_1}{a'_2}$$

where a'_1 and a'_2 are the mean ionic activities of KCl in l_o and l. Since these must be equal to the mean ionic activities[9] a_1 and a_2 in the external solutions we may put $a'_1 = a_1 = \gamma\, c_1$ and $a'_2 = a_2 = \gamma\, c_2$ (where c_1 and c_2 are the external concentrations and γ is the activity coefficient) so that we have (the observed value being 54.7 mv.)

$$0.0547 = 0.058 \frac{u - v}{u + v} \log \frac{(0.899)\, 0.01}{(0.965)\, 0.001}$$

This gives $u \div v = 73.24$, and putting for convenience[10] $v = 1$ we have for the mobility of K^+ in X, $u_K = 73.24$.

In the same manner we have[9] for NaCl (where the observed P.D. is 20.9 mv.)

$$0.0209 = 0.058 \frac{u - v}{u + v} \log \frac{0.903\, (0.01)}{0.966\, (0.001)}$$

from which u_{Na}, the mobility of Na^+ in X, is 2.18.

There is a great difference in the significance of these values. It is evident from Fig. 4 that a considerable error in measuring the P.D. of the concentration effect will not make much difference in u when the P.D. is small, but it makes a very great difference as the P.D. approaches 56.215 (the theoretical maximum for KCl, allowing for activities). Hence the value of u_{Na} is reliable but that of u_K is not. The latter, however, may be satisfactorily obtained from the chemical effect, as

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[8] Similar calculations have been made for *Valonia*. *Cf.* Damon, E. B., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 445.

[9] The activity coefficients are taken from Scatchard, G., *J. Am. Chem. Soc.*, 1925, **47**, 648.

[10] It may be noted that we should not expect to be able to measure diffusion potentials in a non-aqueous layer (such as X presumably is) where the concentration of ions is probably small, were it not for the fact that considerable difference exists between u and v.

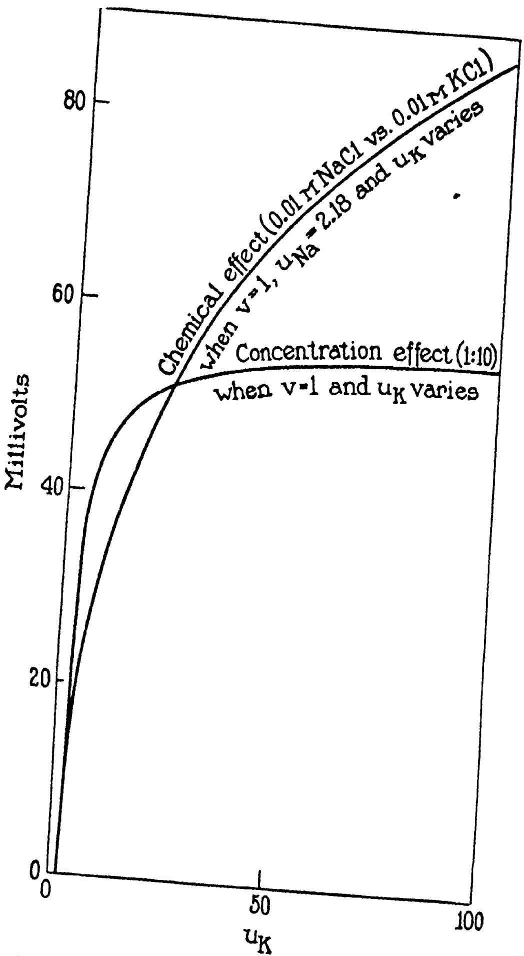

FIG. 4. Calculated concentration effect for tenfold dilution of KCl when $v = 1$ and u_K varies, and calculated chemical effect of 0.01 M NaCl against 0.01 M KCl when $v = 1$, $u_{Na} = 2.18$, and u_K varies.

is evident from Fig. 4 for the observed[11] P.D. of 0.01 M NaCl *vs.* 0.01 M KCl is 82.9 mv. and in this region the slope of the curve is such as to give reliable values.

We may employ the equation of Planck or that of Henderson, both of which in this case reduce to the same formula. If the concentration of KCl in the surface of X is equal to that of NaCl (and the temperature 19°C.) the formula is[12]

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[11] The measurements were made as in determining the concentration effect of KCl, using 0.01 M NaCl in place of 0.001 M KCl, so that a_1 becomes the activity of 0.01 M NaCl.

$$0.0829 = 0.058 \log \frac{u_K + v}{u_{Na} + v} = 0.058 \log \frac{u_K + 1}{2.18 + 1}$$

This gives $u_K = 85.45$.

If we calculate the P.D. of 0.01 M KCl *vs.* 0.001 M KCl by means of this value we have

$$\text{P.D.} = 0.058 \frac{u_K - v}{u_K + v} \log \frac{8.99}{0.965}$$

$$= 0.58 \quad \frac{85.45 - 1}{85.45 + 1}$$

$$= 54.9$$

The observed value is 54.7.

This may be checked by measuring the P.D. of KCl 0.01 M against mixtures of Na and K. We may employ Henderson's formula,[13] which in this case reduces to

$$\text{P.D.} = 0.058 \log \frac{U_{\text{I}} + V}{U_{\text{II}} + V}$$

where $U_{\text{I}} = (u_K)$ (conc. K^+ in Sol. I) + (u_{Na}) (conc. Na^+ in Sol. I), $U_{\text{II}} = (u_{Na})$ (conc. Na^+ in Sol. II), and $V = (v_{Cl})$ (conc. Cl^-).[14]

When Solution I contains 0.005 M KCl + 0.005 M NaCl, and Solution II contains 0.01 M NaCl we have $U_{\text{I}} = (85.45)\ (0.005) + (2.18)\ (0.005)$, $U_{\text{II}} = (2.18)\ (0.01)$, and $V = (1)\ (0.01)$ so that we have

$$\text{P.D.} = 0.058 \log \frac{0.4273 + 0.01}{0.0218 + 0.01} = 66.0 \text{ mv.}$$

The observed value is 68.8 mv.

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[12] For a brief discussion of Henderson's and Planck's formulae the reader may be referred to Michaelis, L., Hydrogen ion concentration, Baltimore, 1926.

[13] This involves the assumption that the concentration of K^+ in X bears the same relation to that of Na^+ as in the aqueous solution. There is no harm in making this tentatively. It also involves the assumption that the electrolyte concentration is the same throughout the boundary between NaCl in X and KCl in X, as is the case at the very start before diffusion has had time to accomplish much.

[14] The concentration of Cl^- is the same in both solutions.

It may be remarked in passing that there is a theoretical advantage in measuring the system $a'_2 \mid a'_1$ (successive solutions on the same spot) rather than $a'_2 \mid a'_3 \mid a'_1$ (different solutions on different spots) for it is well known from experiments *in vitro* that in general we should expect different values in the two cases (and this is in accord with Henderson's equation).[14a] This of course would not apply in the case of phase boundary potentials.

The equations of Planck and of Henderson take no account of activities but this is remedied by some of the more recent formulae. If, for example, we could assume that (as in the case of Henderson's equation) the electrolyte concentration is the same throughout the boundary between NaCl in X and KCl in X (as is the case at the very start before diffusion has had time to accomplish much) and that at each concentration ionic activities and conductances are approximately independent of the accompanying (monovalent) ions, the partition coefficient and dissociation in X being approximately the same for NaCl and KCl, we might adopt the equation used by MacInnes and Jones[15] for aqueous solutions and write

$$FdE = RT\,(T_{\mathrm{Na}}d \ln a'_{\mathrm{Na}} + T_{\mathrm{K}}d \ln a'_{\mathrm{K}} - T_{\mathrm{Cl}}d \ln a'_{\mathrm{Cl}})$$

in which T_{Na}, T_{K} and T_{Cl} are the transference numbers of the ions for mixtures in X. According to MacInnes this leads to the equation (at 19°C.)

$$E = 0.058 \log \frac{(1 - N_{\mathrm{K}}) - x_2(N_{\mathrm{Na}} - N_{\mathrm{K}})}{(1 - N_{\mathrm{K}}) - x_1(N_{\mathrm{Na}} - N_{\mathrm{K}})}$$

where N_{Na} and N_{K} are the transference numbers of the ions in solutions of the pure salts in X, and x_1 and x_2 are the molar proportions of NaCl in the external aqueous solutions (and by assumption in l_0 and l).

[14a] In *Nitella* there is often no great difference between the two measurements.

[15] MacInnes, D. A., *J. Am. Chem. Soc.*, 1921, **43**, 1217. MacInnes, D. A., and Yeh, Y. L., *J. Am. Chem. Soc.*, 1921, **43**, 2563. MacInnes, D. A., and Jones, P. T., *J. Am. Chem. Soc.*, 1926, **48**, 2831. MacInnes, D. A., and Cowperthwaite, I. A., *Trans. Faraday Soc.*, 1927, **23**, 400. Smith, E. R., *Bureau of Standards J. Research*, 1929, **2**, 1137.

Some of the assumptions mentioned in the text may be unnecessary.

When NaCl is absent from one solution so that x_1 (as in the present case) becomes zero we have

$$E = 0.058 \log \frac{(1 - N_K) - x_2(N_{Na} - N_K)}{1 - N_K}$$

The transference number of Na^+ is $2.18 \div 2.18 + 1 = 0.6858$ and that of K^+ is $85.45 \div 85.45 + 1 = 0.98839$. Substituting these values we have for 0.01 M NaCl *vs.* 0.01 M KCl (where $x_2 = 1$)

$$\text{P.D.} = 0.058 \log \frac{(1 - 0.98839) - (0.6858 - 0.98839)\, x_2}{1 - 0.98839}$$

$$= 0.058 \log (1 + 26.063\, x_2)$$

$$= 83.08 \text{ mv.}$$

For 0.01 M NaCl *vs.* 0.005 M KCl + 0.005 M NaCl (where $x_2 = 0.5$) we have

$$\text{P.D.} = 0.058 \log (1 + (26.063)\,(0.5))$$

$$= 65.62 \text{ mv.}$$

Both of these are in close agreement with the other calculations.

These calculations may be checked by considering relative conductances which may be calculated by means of the formula

$$\text{Relative conductance}^{16} = \frac{\alpha C'_{KCl}\,(u_K + v)}{\alpha C'_{NaCl}\,(u_{Na} + v)}$$

where α is the degree of dissociation and C' is the concentration in X. Assuming these to be approximately the same[17] for KCl and NaCl we have

$$\text{Relative conductance} = \frac{85.45 + 1}{2.18 + 1} = 27.19$$

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[16] As usually employed this is specific conductivity but for comparative purposes as in this case two conductances under the same conditions may be used.

[17] If the values of C'_K and C'_{Na} are equal it does not follow that the concentration in X of undissociated molecules (which may be the form in which penetration chiefly occurs) is equal since the dissociation constants may differ. For this reason and also because permeability may be determined by Y rather than X we need not look for a close correspondence between P.D. and permeability.

According to Blinks[6] the observed value[18] in the most reliable range (about 0.1 M) is from 25 to 50 (*i.e.*, the resistance per square centimeter in contact with 0.1 M KCl is 4000 ohms and in contact with 0.1 M NaCl is 100,000 to 200,000 ohms).

Calculation of Phase Boundary Potentials

Let us now consider calculations on the basis of phase boundary potentials alone. For the P.D. of 0.01 M NaCl *vs.* 0.01 M KCl we have

$$E = Pb_2 - Pb_1$$

where Pb_2 is the phase boundary potential of 0.01 M KCl at the outer surface of X and Pb_1 that of 0.01 M NaCl. If we employ the usual equations[19] (which take no account of activities and are of very doubtful validity) we may put (since the observed P.D. is 82.9 mv.)[20]

$$0.0829 = \frac{0.058}{2} \log \frac{A_{\mathrm{K}}}{A_{\mathrm{Na}}}$$

where A_{K} and A_{Na} are the "true" partition coefficients[19] of K^+ and Na^+ between the external solution and X. From this $A_{\mathrm{K}} \div A_{\mathrm{Na}} = 722.27$ and we may for convenience put $A_{\mathrm{K}} = 722.27$ and $A_{\mathrm{Na}} = 1$.

For 0.01 M NaCl *vs.* 0.005 M KCl + 0.005 M NaCl we should then have[20]

$$\text{P.D.} = 0.029 \log \frac{A_{\mathrm{K}}C_{\mathrm{K}} + A_{\mathrm{Na}}C_{\mathrm{Na}}}{A_{\mathrm{Na}}C_{\mathrm{Na}}}$$

$$= 0.029 \log \frac{(722.27)\,(0.005) + (1)\,(0.005)}{(1)\,(0.01)}$$

$$= 74.2 \text{ mv.}$$

The observed value is 68.8 mv.

The concentration effect presents greater difficulty. It is well known that no concentration effect is to be expected from phase boundary potentials unless we make special assumptions. Were this done as suggested by Beutner,[21] for example, we should expect the same concentration effect for KCl as for NaCl. This is not true of *Nitella* where the concentration effect of NaCl is much smaller, as would be predicted on the basis of diffusion potentials since KCl is strongly negative to NaCl.

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[18] It should be remembered that the measurements by Blinks have to do with the resistance across the whole protoplasm while the calculations of mobility may apply only to X.

[19] *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1927–28, **11**, 83.

[20] In this calculation concentrations are employed instead of activities merely for convenience.

If, however, we assume that the "true" partition coefficients vary with concentration as well as with the nature of the ion we might avoid this difficulty, but we should be obliged to assume great variation. For example, if the "true" partition coefficient of K^+ at the concentration 0.1 M be called $A_{K\,0.1}$ and that at the concentration 0.001 M be called $A_{K\,0.001}$ and if the observed P.D. between 0.1 and 0.001 M KCl be[1] 116 mv. (A_{Cl} being constant) we have

$$\text{P.D.} = 0.116 = 0.029 \log \frac{A_{K\,0.1}}{A_{K\,0.001}}$$

whence[22] $A_{K\,0.1} \div A_{K\,0.001} = 10{,}000$.

To calculate the relative conductance we might assume as a first approximation that the degree of dissociation and the mobilities in X are about the same for KCl and NaCl and we should then have

$$\text{Relative conductance} = \frac{\alpha C'_K\,(u_K + v)}{\alpha C'_{Na}\,(u_{Na} + v)}$$

$$= \frac{C'_K}{C'_{Na}}$$

where C'_K is the concentration of K^+ and C'_{Na} that of Na^+ in X. Using the equations given above the value of $C'_K \div C'_{Na}$ may be obtained as follows: we have[19]

$$\text{P.D.} = 0.058 \log \sqrt{\frac{A_K}{A_{Na}}}$$

$$= 0.058 \log \frac{C_K A_K}{C'_K} \; \frac{C'_{Na}}{C_{Na} A_{Na}}$$

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[21] Beutner, R., Die Entstehung elektrischer Ströme in lebenden Geweben, Stuttgart, 1920. Michaelis, L., Hydrogen ion concentration, Baltimore, 1926. Osterhout, W. J. V., *J. Gen. Physiol.*, 1927-28, 11, 83.

[22] This value would, of course, be smaller if A_{Cl} varied in the opposite way from A_K.

where C_K is the concentration of K+ in the external solution and C'_K its concentration in X, etc. Since $C_K = C_{Na}$ we have

$$\sqrt{\frac{A_K}{A_{Na}}} = \frac{A_K}{A_{Na}} \frac{C'_{Na}}{C'_K}$$

Dividing both sides by

$$\sqrt{\frac{A_K}{A_{Na}} \frac{C'_{Na}}{C'_K}}$$

we have

$$\frac{C'_K}{C'_{Na}} = \sqrt{\frac{A_K}{A_{Na}}}$$

In the present case we have

$$\text{Relative conductance} = \frac{C'_K}{C'_{Na}} = \sqrt{\frac{A_K}{A_{Na}}} = \sqrt{722.27} = 26.88$$

It is evident that we can approach the observed value of 25 to 50 by putting u_{Na} approximately equal to u_K, but this seems improbable in view of the fact that K and Na differ so widely in their behavior toward protoplasm.

Calculation of Mixed Potentials

It is clear from all that has preceded that the calculations are very satisfactory if we consider that phase boundary potentials play such a subordinate rôle that they may be neglected and this would be in harmony with recent theoretical tendencies.[23]

If, however, we grant that there may be cases where both phase boundary potentials and diffusion potentials play an important rôle it may be of interest to see what values are obtained by assigning equal weights to phase boundary potentials and diffusion potentials by putting $Pb_2 - Pb_1 = Pd_2$ in the equation $E_{conc.} = Pb_2 - Pb_1 + Pd_2$ (which gives the value of the concentration effect). For 0.01 M *vs.* 0.001 M KCl we should then have $0.0547 = Pb_2 - Pb_1 + Pd_2$ whence $Pd_2 = \frac{0.0547}{2} = 0.02735 = \frac{u_K - 1}{u_K + 1}(0.058) \log \frac{8.99}{0.965}$, whence $u_K = 2.895$ and in similar fashion we obtain $u_{Na} = 1.456$. We may call the phase boundary potential, when the protoplasm is in contact with 0.01 M NaCl, Pb_3 and put

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[23] Guggenheim, E. A., *J. Phys. Chem.*, 1929, **33**, 842. Cremer, M., *Handb. norm. u. path. Physiol.*, 1928, **8**, pt. 2, 1034 (especially the remark concerning Debye).

$$Pb_2 - Pb_3 = \frac{0.0829}{2} = 0.029 \log \frac{A_K}{A_{Na}}$$

whence $A_K \div A_{Na} = 26.87$ or putting $A_{Na} = 1$ we have $A_K = 26.87$.

Using these figures we may calculate the P.D. of 0.01 M NaCl *vs.* 0.005 M NaCl + 0.005 M KCl. We then have

$$\text{P.D.} = 0.058 \log \frac{U_I + V}{U_{II} + V} + 0.29 \log \frac{C_K A_K + C_{Na} A_{Na}}{C_{Na} A_{Na}}$$

$$= 0.00648 + 0.0419$$

$$= 48.4 \text{ mv.}$$

The observed value is 68.8 mv.

To calculate the relative conductance we should have (assuming the degree of dissociation of KCl in X to be about the same as that of NaCl)

$$\text{Relative conductance} = \frac{C'_K(u_K + v)}{C'_{Na}(u_{Na} + v)}$$

$$= \sqrt{26.87}\left(\frac{3.895}{2.456}\right)$$

$$= 8.22$$

Since the observed value is 25 to 50 it is evident this method of calculation is unsatisfactory.

The assumption that phase boundary potentials are as important as diffusion potentials in the concentration effect does not appear to be a fortunate one.

DISCUSSION

It is clear that for purposes of calculation it is much better to proceed as if diffusion potentials predominated. As the ionic mobilities found by these calculations are unlike those in water the nature of the protoplasmic surface becomes an important question. This surface is usually regarded as liquid since protoplasm in contact with water (either occurring naturally or when squeezed out of the cell by pressure) commonly rounds up like an oily liquid, but this might occur if it were covered with a very thin solid film, comparable to a drop of mercury with a film of oxide on its surface. Plant protoplasm which secretes cellulose may possibly have on its outer surface a very thin film (solid or semisolid) of something like cellulose, which may adhere to the protoplasm and pull away from the cell wall during plasmolysis (but such a film seems much less probable in the case of the inner protoplasmic surface which surrounds the vacuole in most plant cells).

The mention of cellulose raises the question whether the cell surface acts like certain collodion membranes whose P.D.'s (somewhat resembling those of *Nitella*) are, according to Michaelis,[24] explainable on the basis of diffusion potentials. (Collander,[25] and Northrop[26] state that these membranes resemble the protoplasmic surface in some other respects.)

As nothing could be more welcome than a satisfactory model of the cell surface it may be desirable to inquire whether collodion really suffices for this purpose. Aside from the necessity of supposing the cell surface to be always solid there are difficulties. The concentration effects, for example, which are almost identical for HCl, KCl, NaCl, and LiCl with collodion, differ greatly[27] in *Nitella*. For collodion the ionic series is the same as with water (as far as investigated: Cs has not been studied); if this applied to *Nitella* each ion in the series Cs, Rb, K, Na, Li should be positive to all those preceding it and negative to all those following it. This is by no means the case: for example K and Rb are[28] strongly positive to Cs, and likewise tetraethyl ammonium fails to show the positivity to Na which is expected.

The fact that the ionic series is not the same as for water would be intelligible if the outer protoplasmic surface were non-aqueous and we should expect the order to be an inverse function of the atomic weights unless disturbed by attractions between the ions and the non-aqueous phase producing results similar to hydration in aqueous media.

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[24] Michaelis, L., and others. Articles in *J. Gen. Physiol.*, 1925-29, **9-12**. Molecular physics in relation to biology, *Nat. Research Council Bulletin*, 1929, No. 69, 119.

[25] Collander, R., *Kolloidchem. Beih.*, 1924, **19**, 72; 1925, **20**, 273; *Soc. sci. Fennica, commentationes biol.*, (6) 1926, **2**, 1.

[26] Northrop, J. H., *J. Gen. Physiol.*, 1927-28, **11**, 233; 1928-29, **12**, 435; 1929-30, **13**, 21.

[27] This will be discussed in later papers.

[28] A similar effect is reported for muscle by Höber, R., Physikalische Chemie der Zelle und der Gewebe, Leipsic, 6th edition, 1926, 650.

In this connection we need not detail all the features which must exist in a non-living model before it can be called completely satisfactory, but it may be desirable to emphasize certain fundamentals.

(1) The surface of a growing cell must admit both anions and cations or undissociated molecules of electrolytes. Höber[29] suggests that the surface of such cells is comparable to a mosaic of positive and negative collodion membranes: but how could such a mosaic be created? Is it not simpler to suppose that the protoplasmic surface is a non-aqueous phase through which salts pass by forming molecules, just as HCl passes through air from one aqueous solution to another by temporarily forming molecules? In this respect air may act in somewhat the same way as the protoplasmic surface but various non-aqueous substances, such as amyl alcohol, may furnish a closer analogy. Such formation of molecules would permit only a slow passage but certain substances with molecules preformed in the solution (*e.g.*, NH_3, H_2S, CO_2) show a more rapid passage through air, amyl alcohol, and protoplasm. But not all molecules pass out with equal facility; for example, we observe (with air, with amyl alcohol, and with protoplasm) a ready passage of alcohol but not of sugar.

The chemical and concentration effects indicate that certain ions enter X and it is of course possible that ion pairs, such as $Na^+ + Cl^-$, penetrate rapidly enough to play an important rôle in permeability or that H^+ and organic anions, produced in the cell, are exchanged for Na^+ and Cl^-, but the high electrical resistance of the protoplasm for most ions, observed by Blinks,[6,30] does not favor this idea. If the outer surface of the protoplasm is non-aqueous (lipoid, for example) it would presumably contain a higher concentration of molecules than of ions so that (unless the mobility of ions greatly exceeds that of molecules) penetration would be largely in molecular form.

(2) Subjecting normal cells of *Nitella* or *Valonia* to an electric

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[29] Höber, R., and Hoffmann, F., *Arch. ges Physiol.*, 1928, **220**, 558.

[30] Blinks, L. R., unpublished results.

current produces a series of effects (*e.g.*, back E.M.F. with direct current, falling off of impedance as a function of the frequency with alternating current) not imitated by collodion or by amyl alcohol or other non-living models except perhaps by metallic electrodes.[6]

(3) In many cases the protoplasmic surface undergoes great and rapid changes in permeability with complete or partial recovery which have not yet been satisfactorily imitated by any model.

In view of these and other complications the question of a model appears to need further investigation.

SUMMARY

The P.D. of 0.01 M *vs.* 0.001 M KCl (concentration effect) in *Nitella* is 54.7 mv. and that of NaCl is 20.9 mv. The P.D. of 0.01 M NaCl *vs.* 0.01 M KCl is 82.9 mv.

If we assume that diffusion potentials predominate we may calculate the relative mobilities of the ions in the outer layer of the protoplasm. If we put $v_{Cl} = 1$ we obtain $u_K = 85.45$ and $u_{Na} = 2.18$. (These values depend upon the fact that the mean ionic activities in this layer must be equal at equilibrium to the mean ionic activities in the external solution.)

Using these values the calculated P.D. of 0.01 M NaCl *vs.* 0.005 M NaCl + 0.005 M KCl is 66 mv. (the observed value is 68.8 mv.) and the conductance of KCl in the outer layer of the protoplasm is 27 times as great as that of NaCl (the observed conductance for the whole protoplasm is 25 to 50 times greater for KCl than for NaCl).

Calculations on the basis of phase boundary potentials are less satisfactory.

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A METHOD FOR THE QUANTITATIVE ESTIMATION OF BACTERIA IN SUSPENSIONS

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Determinations of the number of bacterial cells present in a given suspension may be accomplished by several different means. Besides the classical plate and dilution methods which have contributed so much to the development of bacteriology, there are suitable procedures for direct microscopical counts, using either a counting chamber or a stained mount. Further, in certain instances, the total cell concentration of a suspension may be estimated by nephelometric comparison, by weighing the washed and dried sediments obtained from centrifuged aliquots, or by measuring a product of metabolism.

For some types of work the volume occupied by organisms has proven a satisfactory measure of the number of cells. Total cell volume is determined by centrifuging, the sediment being deposited in a calibrated cup from which the volume of packed cells is read directly. Having already established the volume-cell number ratio, preferably by direct count, volume readings are readily convertible into total cells per cubic centimeter.

Paine (1), Carlson (2), and Slator (3) have reported favorable experiences with such a technic used by each of them to determine the cell content of yeast cultures, while Hopkins (4) has successfully utilized the method for standardizing bacterial vaccines.

During the course of a study of bacteriophage action on staphylococci there arose a two-fold necessity: first, for a method by which estimates of cell numbers in bacteriophage-containing suspensions might be obtained; and second, for a technic giving rapid and accurate cell number determinations in dense preparations to be used for making up dilutions of known bacterial concentrations. Obviously, the plate and dilution methods could not be employed in the presence of bacteriophage, and neither procedure would furnish immediate information for preparing cell dilutions. It was finally found possible to adapt the centrifuged-sediment method to these problems with satisfactory results.

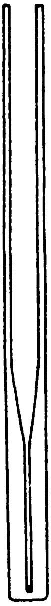

FIG. 1

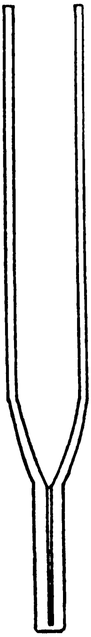

FIG. 2

FIG. 1. Small centrifuge tube for dense suspensions. Length: 11 cm. Outside diameter: 8 mm. Length of capillary: 25 mm. Capillary bore: 1 mm. Small drop of mercury at bottom to give clear meniscus.

FIG. 2. Large centrifuge tube for light suspensions. Length: 13 cm. Outside diameter: 1.7 cm. Length of capillary: 30 mm. Capillary bore: 0.6 mm. Small drop of mercury at bottom to give clear meniscus.

The centrifuge tubes (Fig. 1) constitute the essential feature of the method. They are calibrated individually with dense cell suspensions which are made up in buffer solution, and standardized by direct count. Five consecutive 1 ml. aliquots are centrifuged at known speed for a period of time sufficient to insure maximal packing. The column length of the sediment is measured in each instance with a cathetometer or vernier calipers and the average bacterial content per millimeter of tube length calculated. Both menisci are level and sharp and permit accurate measurement.

Tubes calibrated in this way were employed in actual routine as follows: 16 hour cultures of a single strain of *Staphylococcus aureus* grown in Blake flasks were washed from the agar with $\frac{M}{200}$ phosphate buffer of pH 7.6 using 30 ml. to each flask. The emulsion was filtered through a sterile Schleicher and Schüll Faltenfilter No. 588 and 1 ml. aliquots centrifuged in the small tubes (Fig. 1) at 2600 R.P.M. for 15 minutes. The lengths of the columns of packed bacterial cells were then measured and the figures converted into cells per milliliter of original suspension. From such suspensions dilutions of any required cell content were of course readily prepared.

The difference between consecutive measurements of the same suspension was found to be <1.0 per cent (triplicate determinations on 65 emulsions), while the total error based on repeated checks with careful direct counts was consistently <2 per cent. These figures apply to the measurement of very dense suspensions containing approximately 14×10^9 cells/ml. and giving a column length of about 20 mm.

In the case of light suspensions of staphylococci in nutrient broth, 10 ml. aliquots added to 2 ml. of a mixture of acetone 10 ml. $\frac{M}{1}$ NaCl 3.0 ml., neutral formalin 2.0 ml., were centrifuged at 2600 R.P.M. for 15 minutes in large cups (Fig. 2). These cups were calibrated with light suspensions of known cell content (direct count), which were mixed with the acetone-salt-formalin reagent before centrifuging. Here a larger percentage of the organisms remained in the supernatant fluid and the difference between successive determinations on the same suspension was greater than in the case of the dense preparations, averaging approximately 4 per cent. The total error based on direct count figures averaged 5 per cent.

SUMMARY

A method is described for estimating the cell concentration of bacterial suspensions by measurement of the column lengths of packed

cells centrifuged into a capillary tube of fine bore. The total error, under the experimental conditions employed, checked by direct count, was <2 per cent for dense suspensions and about 5 per cent for light suspensions. The method is suitable for work requiring rapid and accurate routine preparation of bacterial suspensions of known cell concentrations.

The writer wishes to express his indebtedness to Dr. John H. Northrop for generous aid and guidance and to Miss Virginia Toussaint for faithful technical assistance.

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A METHOD FOR THE QUANTITATIVE DETERMINATION OF BACTERIOPHAGE

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Two general methods for the quantitative determination of bacteriophage are known. The first of these involves testing serial dilutions of the lytic agent for ability to produce complete dissolution of a broth culture of susceptible bacteria. The last effective dilution is divided into several aliquots which are in turn seeded with phage-susceptible organisms. Thus if four 1 ml. portions of a 10^{-6} dilution of a phage suspension produce lysis and six do not, it is assumed that each of the four active samples contains at least 1 lytic particle and that therefore the original lysate possesses a minimum concentration of 4×10^6 phage corpuscles/ml.

The alternate method consists of plating appropriate phage dilutions upon a substrate of susceptible bacteria. The round, punched-out lytic areas are considered to represent the loci of single phage particles, and a count of their numbers should furnish a simple measure of the total phage per milliliter.

D'Herelle has fully described and discussed both procedures and apparently has been able to obtain satisfactory checks with them. In judging the suitability of these technics for critical quantitative work, however, it would appear that even in d'Herelle's hands agreement of results obtained by the two methods has been wanting. To quote d'Herelle (1) "The undiluted suspension . . . . of the more powerful race contained 200,000 million (2×10^{11}) corpuscles per cc. according to the dilution method and 121,000 (1.21×10^{11}) according to the plaque method. For the second race the number of corpuscles was 6×10^9 by the first method and 8.1×10^9 by the second." The extreme possible errors in the cited cases are 65 per cent and 35 per cent respectively.

Clark (2) has analyzed the dilution method of titration from the statistical viewpoint and concludes that with a dilution constant of 0.1 only about 60 per cent of parallel runs on the same solution should give the same end-point. It is apparent that a difference of 1 tube in the end-point of a series of this sort is apt to produce a considerable error in the final calculation. A recent survey by the writer of previous titration data of his own has given a figure but little greater than the 60 per cent of Clark's.

Plaque counts appear to offer no particular advantage over the dilution technic as far as agreement between parallel runs is concerned. Several factors other than the concentration of phage have been shown to influence markedly the number of plaques appearing on solid media (3) and considerable personal experience with the method has led the writer to the conclusion that, in his hands at least, it is inadequate for accurate determinations.

In attempting to devise some more suitable technic the reaction between a highly active antistaphylococcus phage and a single strain of *S. aureus* was studied. Viscosimetric measurements of bacterial swelling following the addition of phage to dense suspensions failed to furnish any basis for quantitative phage estimations. Determinations of the number of bacteria required to completely adsorb phage from exposed lysates resulted similarly.

As a next step a study of the effects produced by varying the C_{Phage} and $C_{\text{Bact.}}$ present in a given volume at the beginning of the reaction was undertaken. Although it is stated (4) that "within certain very wide limits the quantity of bacteriophage filtrate necessary to inoculate to cause this dissolution is a matter of no moment; the course of the action and the final result of the phenomenon is the same, whether the amount inoculated is 1 cc. or 0.001, to 10 cc. of medium," this was not found to be the case in the present instance.

Marked differences in the course of the reaction were found to occur upon varying the concentrations of the reactants. For example, under the experimental conditions described below, 4 ml. of 1 per cent phage added to 1 ml. of a broth suspension of staphylococci containing a total of 12.5×10^7 cells produced clearing in 1.9 hours, whereas a similar preparation made with 0.01 per cent phage required 3 hours for the completion of lysis. Comparable results over a wide range of phage concentrations in a constant total volume of 5 ml. containing 12.5×10^7 cells suggested that under these conditions lysis is a function of C_{Phage} and time, and that quantitative estimations of phage could be made either by determining the length of time required to produce clearing of a culture containing a certain number of bacteria at the beginning of the process or by determining the number of bacteria lysed in a given time. Upon this general basis the following procedure was developed.

Details of Method for Quantitative Phage Determination as Used in Present Work with S. aureus and Antistaphylococcus Phage

A considerable quantity of phage is prepared by adding bacteriophage and susceptible organisms to nutrient broth of pH 7.6 in such amounts that respective concentrations of 1.0 per cent phage and 2.0×10^7 cells/ml. are obtained. The mixed reactants are rocked at 36°C. for 3 hours by which time lysis is complete. The lysate is stored in liter flasks at 5°C. *without filtering.* Under such conditions it has been found to maintain full strength satisfactorily over a period of months.

Units: Bacteriophage, like enzymes, can only be measured by its activity and the units employed therefore will have the dimensions of a velocity constant; *i.e.* in this case the number of bacteria lysed per unit of time per unit of phage. The rational unit would be that quantity which would lyse one bacterium in one second but since this cannot be determined experimentally it is necessary to choose an arbitrary number. The rate of lysis varies with temperature, pH, and condition of the culture; consequently these variables must also be fixed. The unit of phage used in this paper is defined as the minimum quantity which will cause complete clearing of 1.25×10^8 cells of *S. aureus* (prepared from a 16 hour culture grown on agar) in 5 ml. pH 7.6 broth at 36°C. 1×10^{-10} ml. of the "standard" phage suspension prepared as described above suffices to produce lysis under these conditions and therefore contains 1×10^{10} units per ml. On this basis a 1 per cent phage suspension will contain 1×10^8 units/ml., a 0.01 per cent suspension 1×10^6 units/ml., etc. The working of this scale in practise is brought out below.

TABLE I

Amounts of S. aureus and Antistaphylococcus Phage Used in Quantitative Phage Determinations

$c_{\text{Bact.}}$ in suspension	Amount of bacterial suspension used	c_{Phage} used		Amount of phage dilution used	$c_{\text{Bact.}}$ in mixture
		per cent	*units*		
12.5×10^7/ml.	1 ml.	0.01	1×10^6	4 ml.	2.5×10^7/ml.
"	"	.001	1×10^5	"	"
"	"	.0001	1×10^4	"	"

A 16 hour culture of the test organism grown in Blake flasks is washed from the surface of the agar with saline solution. The resulting emulsion is filtered through a Schleicher and Schüll Faltenfilter No. 588 and its bacterial content determined by the centrifuged-sediment method (5). Dilutions in broth are immediately prepared and thoroughly mixed with standard phage dilutions in the amounts shown in Table I. Tubes of uniform diameter and any convenient size are employed. We regularly use large tubes of 15 mm. inside diameter because of the accuracy with which they may be read. They are placed in a mechanical shaker mounted in a water bath maintained at 36°C. and are read at short intervals as described below.

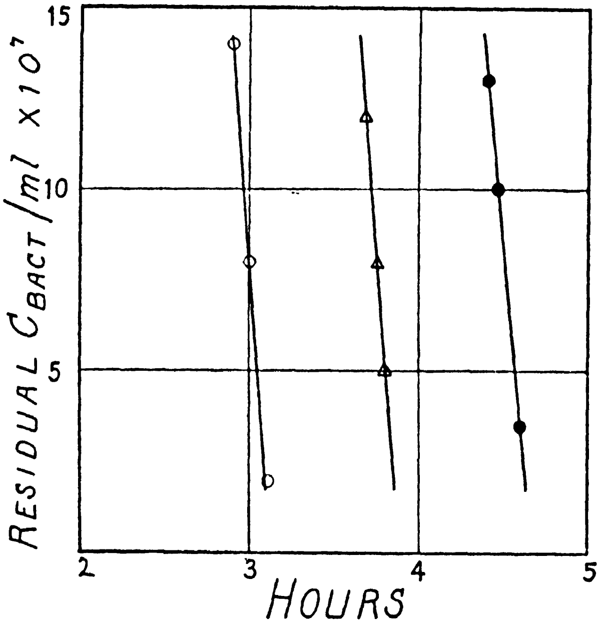

FIG. 1. Residual bacterial concentrations during lysis by various amounts of phage plotted against time

○ 1 × 10^6 Units Phage
△ 1 × 10^5 " "
● 1 × 10^4 " "

Readings are made by comparing the turbidity of the lysing suspension with formalinized bacterial suspensions of known cell concentrations contained in standard tubes. A series of 1, 2, 3, 4, 5, 6, 8, 10, 12, 15, and 20 × 10^7 staphylococci/ml. in broth containing 5 per cent formalin is prepared each day for this purpose.

The light source for reading is important. A satisfactory arrangement is made by mounting a bullet-shaped daylight microscope lamp just below the level of the eyes, turning it at an angle of 45° with the vertical. The tubes are held in front of and slightly below the daylight filter so that they are illuminated against a dark background. Considerable Tyndall effect is obtained in this way and suspensions of 9 × 10^7 and 10 × 10^7 staphylococci/ml., for example, may be readily differentiated.

Growth of bacteria occurs in all the tubes and turbidity increases greatly. Readings are begun when the first diminution in turbidity becomes apparent and are recorded graphically by plotting the residual bacterial concentrations as ordinates against the time required to reach these values. Curves of the type indicated in Fig. 1 are obtained. On such curves one chooses a convenient end-point (here 8 × 10^7 Bact./ml.) and interpolates a second curve, Fig. 2, by plotting the logs of the phage units required to bring the original bacterial suspension to this arbitrary end-point against the time spent in the process. Both sets of curves are reproducible and may be quite closely fixed. However, in titrating unknowns it is best to include two known phage dilutions in each set-up. Readings from the control tubes may shift the second curve slightly but will not alter the slope.

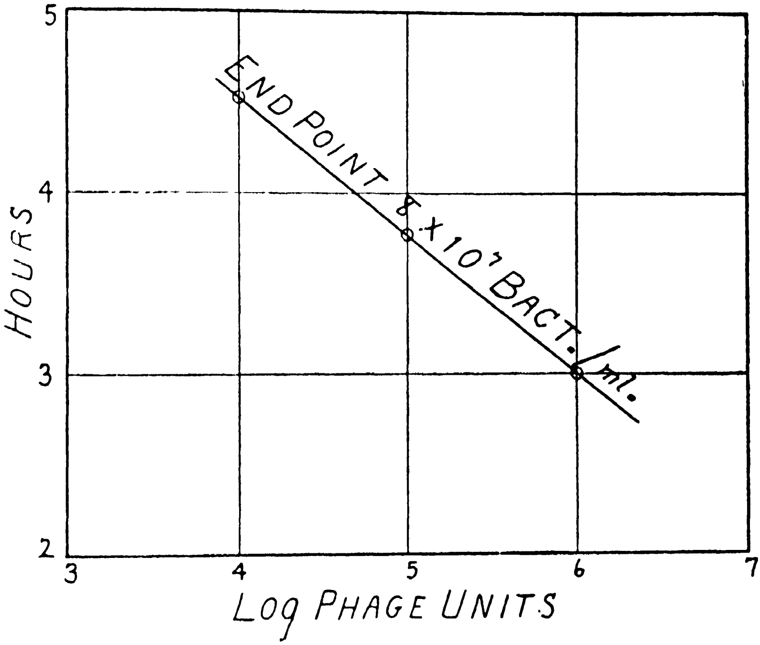

FIG. 2. Log of C_{Phage} (units) plotted against the time required to reduce $C_{\text{Bact.}}$ to 8 × 10^7/ml.

An unknown is run as follows. 4 ml. aliquots of the undiluted lysate and four 1:10 serial dilutions in broth are pipetted into standard tubes. To each is added 1 ml. of a broth suspension of staphylococci prepared as described above and containing 12.5 × 10^7 Bact./ml. The tubes are mounted in the water bath shaker and at 2.5 hours readings are begun. Any original C_{Phage} between 100 per cent (1×10^{10} units) and 0.0001 per cent (1×10^{4} units) will give in at least one of the five tubes a sharp decrement in $C_{\text{Bact.}}$ at some time between 3 and 4.5 hours. It is essential to catch this explosive process of lysis so that two or preferably three points may be established for drawing the curve of Fig. 1. In order to be sure of this the tubes are read every 0.2 hour between 2.5 and 4.7 hours. When the turbidity in any tube drops below 20×10^{7} Bact./ml. that tube is read every 0.1 hour. Such a procedure avoids any difficulty apt to arise from insufficient data for fixing the slope of the curve.

From the plot of residual $C_{\text{Bact.}}$ against time one reads the time required to reach a given end-point (here 8×10^{7} Bact./ml.). This time value is located on the second standard curve and the corresponding abscissal figure (Log Phage Units) read directly. Suppose, for example, that a 10^{-3} dilution of an unknown reduces the turbidity of the test suspension to 8×10^{7} in 3.6 hours. The log phage units corresponding to 3.6 hours on the 8×10^{7} curve is 5.2. Therefore, the original unknown contains $1 \times 10^{5.2} \times 10^{3}$ (dilution factor) units per milliliter. Changing to antilogs, this is equivalent to 1.58×10^{8} phage units.

DISCUSSION

From the description given it is obvious that the method rests upon the fact that a set concentration of growing phage-susceptible bacteria contained in a unit volume of phage-bacteria mixture will be reduced to some arbitrary turbidity end-point at a time depending upon the number of phage units present at the start of the process. The amounts of phage involved in the reaction are conveniently expressed by defining the standard phage as containing 1×10^{10} units. It should be stated that this figure is not the actual number of lytic particles in the standard; it is a purely arbitrary constant adopted for convenience. In the actual test 4 ml. of phage or phage dilution are used while titer calculations are based upon 1 ml. quantities. No correction for this difference need be made since the 4 ml. quantity is constant throughout.

The original $C_{\text{Bact.}}$, the turbidity end-point, the total volume, and the C_{Phage} range used for estimating unknowns, are again not set values but are fixed in each instance by preliminary runs with the particular phage and bacterium being studied.

In the present case phage values $<1 \times 10^4$ units/ml. did not give points lying on the curve of Fig. 2. Such irregularity is to be expected in very high dilutions and the range for quantitative estimations must be chosen with this in mind. Similarly, concentrated lysates will not fit the curve because the turbidity of the suspension never develops sufficiently to furnish the required end-point.

The accuracy of the method is well within ±5 per cent and depends to some extent upon the shaking of the tubes. Shaking speeds up the process markedly and decreases the differences noted in repeated runs with unshaken tubes. In the latter case the different degrees of jarring the tubes are given at the time of reading probably account for such minor variations.

It is possible to use still another method for quantitative work. Various dilutions of standard phage are mixed with increasing concentrations of bacteria, maintaining a constant total volume. Some convenient end-point as in the above procedure is chosen and for each particular C_{Phage} the time required to reach this $C_{\text{Bact.}}$ is plotted against the original bacterial concentrations used. From the series of curves so obtained a second curve is interpolated by plotting the original phage values (units) against the concentrations of bacteria which they reduced to the turbidity end-point at some particular time. The second curve furnishes a standard for determining unknowns just as in the method described above in detail.

CONCLUSIONS

1. In the case of the staphylococcus and antistaphylococcus phage studied, with the total volume of the mixture being kept constant, there exists a definite quantitative relationship between C_{Phage} and the time required to reduce a particular concentration of growing phage-susceptible bacteria to an arbitrary turbidity end-point.

2. This relationship furnishes a basis for the quantitative estimation of bacteriophage. A method is described having an accuracy within ±5 per cent.

It is a pleasure to acknowledge the aid and interest of Dr. John H. Northrop in this work and the faithful technical assistance of Miss V. Toussaint.

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ELASTICITY, DOUBLE REFRACTION AND SWELLING OF ISOELECTRIC GELATIN

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The importance of the rôle which elasticity plays in the mechanism of swelling of a gelatin gel is well recognized in Procter-Wilson's theory. of swelling of gelatin in acid or alkali. According to this theory (1) there are two opposing forces acting on a block of gel when placed in an aqueous solution of acid or alkali, first, the osmotic force which is brought about by the excess of diffusible ions in the gel over those in the outside solution and which causes the gel to swell, and second, the elastic force produced by the rigidity of the gel structure and which limits the extent of swelling. At equilibrium the two forces balance each other. Thus the elasticity of the gel plays the same rôle in the swelling of gelatin as hydrostatic pressure in osmosis phenomena.

Procter-Wilson's theory of swelling has been applied by Northrop and the writer (2) to the mechanism of swelling of isoelectric gelatin in the absence of acid or alkali. Swelling in this case is caused by the presence in gelatin of a soluble fraction which produces osmotic pressure. But the swelling in this case also is regulated by the elasticity of the block as a whole. When a gelatin solution is cooled and allowed to set, the gel formed is under no elastic stress, but as soon as it is placed in water and allowed to swell the gel undergoes an elastic strain producing a stress in it which resists the swelling until an equilibrium is established between the two opposing forces.

Both the equilibrium conditions of swelling of isoelectric gelatin (3) as well as the kinetics of swelling (4) have been found to agree quantitatively with the described theory. The peculiar phenomena observed by Arisz (5) and others that gels of various concentrations when dried to an identical high concentration swell rapidly to their original concentrations and then take up water slowly, and also the effect of varying temperatures, were readily explained by Northrop (3) on the basis that the gel at setting is under no elastic stress, but on drying the gels are put under a compressive stress which makes the gels swell rapidly to their original volume when placed in water. A block of gelatin which has swollen to a constant value at a low temperature and has then been removed from the water and kept for about an hour at a higher temperature, continues to swell when replaced in cold water. The raising of temperature destroys the internal stresses in the gel, and allows the gel to swell more under the influence of osmotic forces.

It is thus evident that a knowledge of the elastic properties of gels is as important as that of the osmotic pressure phenomena to a clear understanding of the mechanism of swelling.

A great deal of work has been done on the determination of the elastic constants of gels.[1] In practically all of these studies the observations were made on the elastic properties of gels during mechanical deformation such as stretching, compressing or twisting. But, as stated above, elastic stresses may be induced in a gel by simply allowing it to swell in cold water. Hence attempts were made in a previous work (6) by Northrop and the writer to determine the effect of salts on the bulk modulus of elasticity of gelatin during swelling from data on osmotic pressure and swelling of gelatin in the presence of salts, under the assumption that the osmotic pressure of gelatin solutions is proportional to the swelling pressure.

A method was developed (7) to measure directly the swelling pressure of gels of various gelatin contents. At equilibrium this equals the internal elastic stress. Calculations were thus made of the bulk modulus of elasticity of gels of various concentrations of gelatin at swelling; but this method is quite complicated and subject to many errors.

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[1] A long list of old references on this subject is given by H. Freundlich in his Kapillarchemie, Leipzig, 3rd edition, 1923, 986; and also by Harry B. Weiser in his article on "Jellies and gelatinous precipitates" in Bogue, R. H., The theory and application of colloidal behavior, New York, 1924, **1**, 402. Important studies of the elastic properties of gels have been made recently in this country by S. E. Sheppard and his collaborators, *J. Am. Chem. Soc.*, 1921, **43**, 539; 1922, **44**, 1857; *J. Ind. and Eng. Chem.*, 1923, **15**, 571; 1924, **16**, 593; and also by G. W. Scarth, *J. Phys. Chem.*, 1925, **29**, 1009.

A more direct and simple method, which has been employed in this work, is to determine internal elastic stresses by measuring the double refraction produced in the gel during swelling. This method of measuring internal stresses is employed quite extensively in engineering as a means of studying internal stresses in cases which are difficult to analyze mathematically. "Thus Coker (9) was able by means of loaded transparent zylonite models to verify well-known calculations on stress distribution such as those for hook sections and perforated tie bars, besides very numerous cases of material having discontinuities such as notches of various kinds, where exact mathematical treatment is practically impossible."[2]

Double Refraction in Materials Subjected to Mechanical Stresses

It is not the intention of the writer to enter here into a general discussion of double refraction, a subject which is fully discussed in books on optics and optical crystallography.[3] The few remarks given here have the object only to define the terms and symbols used in the experimental part of this work.

It is known since Brewster's studies (Philos. Trans., 1816) that when a strip of an isotropic material such as glass, resin, or gelatin, is put under a mechanical stress like tension or compression it becomes optically double refractive.

This is observed by placing the strip of material on the stage of a polarizing microscope between crossed Nicol prisms and exerting the tension or compression in a direction of 45° with the directions of the vibration planes of the crossed Nicols. The plane polarized beam of light from the polarizer or lower Nicol is resolved on entering the strained material into two component beams, one of which has its vibrations in a plane parallel to the direction of the applied stress while the other beam has its vibration in a plane perpendicular to it. There is also a difference in the velocity with which these component beams of light travel through the strained material. In the case of most materials the faster beam is in the direction of tension, while if the material is compressed the beam of light whose plane of vibration is in the direction of compression is the slower one.

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[2] Cited from Morley, A., Strength of materials, London, 1920.

[3] An excellent and clear discussion of the whole subject as applied to colloids is given in Ambronn-Frey's little book, Das polarisationsmikroskop, Leipzig, 1926.

On entering the upper Nicol the two beams of light are made to travel along the same path after they have traveled with a different velocity through the doubly refractive medium; and interference results.

Let V_0 be the velocity of the beam of light in air, which is about the same as in a vacuum, and V_1 and V_2 the velocities of the component beams in the doubly refractive medium, V_1 being the velocity of the faster beam. Let also λ_0, λ_1, and λ_2 be the corresponding wave lengths of the beams. Since the color of the light is not affected by passing through the strained colorless material it follows that f, the frequency of vibration, which is the factor that determines color, is the same for the component beams as for the original one.

Hence

$$\lambda_0 = \frac{V_0}{f}$$

$$\lambda_1 = \frac{V_1}{f}$$

$$\lambda_2 = \frac{V_2}{f}$$

or

$$\lambda_1 = \lambda_0 \frac{V_1}{V_0} = \frac{\lambda_0}{n_1}$$

and

$$\lambda_2 = \lambda_0 \frac{V_2}{V_0} = \frac{\lambda_0}{n_2}$$

where n_1 and n_2 are the indices of refraction of the material in the direction of the stress and in the direction perpendicular to it.

The faster beam of light in passing through a distance, s, of the strained material will complete $\frac{s}{\lambda_1} = \frac{s\,n_1}{\lambda_0}$ wave lengths, or vibrations, while the slower beam will complete a greater number of wave lengths in passing through the same distance, s, namely

$$\frac{s}{\lambda_2} = \frac{sn_2}{\lambda_0}$$

Since the time required for one vibration is the same for both beams it follows that the slower beam will be retarded by the time required to complete

$$\frac{sn_2}{\lambda_0} - \frac{sn_1}{\lambda_0} = \frac{s}{\lambda_0}(n_2 - n_1) = \Delta p \qquad \text{(Equation 1)}$$

wave lengths. This retardation or phase difference which is expressed in wave lengths or fractions of a wave length is the cause of the interference colors observed, with white light as a source of illumination, when the two beams are made to pass through the same path through the upper Nicol. The interference colors thus produced serve as an approximate measure of the phase difference between the two beams of polarized light.

The magnitude of double refraction is defined by the value of $n_2 - n_1$, *i.e.*, by the difference between the two indices of refraction. There are various methods for measuring accurately the phase difference in double refraction. The most accurate and convenient to use is the Babinet compensator method, a detailed description of which is given by Johannsen (9).

If light of a known wave length λ_0 is employed then the value of $n_2 - n_1$ is readily determined.

The Relation between Double Refraction and Elastic Strain

The theory of the relation between double refraction and elastic strain in an isotropic body was developed by Neumann (10). He considered the case of a body which is under strain in three dimensions, *i.e.*, under a volume strain. Let a, b, and c be the three dimensions of an isotropic body, and let α, β, and γ, be the strains in the three directions, *i.e.*,

$$\alpha = \frac{\Delta a}{a}, \quad \beta = \frac{\Delta b}{b}, \quad \text{and } \gamma = \frac{\Delta c}{c}$$

then, according to Neumann, the velocity, V_1 of light in the isotropic medium when under no strain is changed to

$$\begin{aligned} A &= V_1 + q\alpha + p\beta + p\gamma \\ B &= V_1 + p\alpha + q\beta + p\gamma \\ C &= V_1 + p\alpha + p\beta + q\gamma \end{aligned}$$

where A, B, and C are the velocities of light in directions of a, b, and c, and q and p are constants depending on the material.

The technique of measuring double refraction determines the differences between the indices of refraction in the medium in directions lying in a plane perpendicular to the direction of observation. If the observation (*i.e.*, the axis of the polariscope) is in the direction of c, then the observed phase difference, Δ_p, is proportional to the difference between the indices of refraction in the directions of a, and b. *i.e.*,

$$\Delta p = \frac{c}{\lambda_0}\left(\frac{V_0}{A} - \frac{V_0}{B}\right)$$

But

$$\frac{V_0}{A} - \frac{V_0}{B} = \frac{V_0(V_1 + p\alpha + q\beta + p\gamma - V_1 - q\alpha - p\beta - p\gamma)}{A \times B}$$

or

$$\frac{V_0}{A} - \frac{V_0}{B} = \frac{V_0(p - q)(\alpha - \beta)}{A \times B}$$

Since $V_1 - A$ and $V_1 - B$ are very small numbers as compared with the values of A, B and V_1, therefore

$$\frac{V_0}{A} - \frac{V_0}{B} = \frac{V_0(p - q)(\alpha - \beta)}{V_1^2}$$

$\frac{V_0}{V_1^2}$ as well as $p - q$ are constant for a given material and a given monochromatic source of light. It follows then that when a body is under a bulk strain the observed double refraction is proportional to the difference in the linear strains in the directions lying in a plane perpendicular to the direction of observation. It also follows that if the body is stretched, or compressed in the same proportion in both directions, *i.e.*, if $\frac{\Delta a}{a} = \frac{\Delta b}{b}$ no double refraction is to be observed.

Double Refraction in Gelatin

When gelatin is subjected to mechanical stresses it shows the same type of double refraction as ordinary glass, namely, that the direction of tension becomes the direction of minimum index of refraction, while the direction of compression is the direction of maximum index of refraction. The relation between elastic tension and double refraction in gelatin was studied in detail by Leick (11). He found that the double refraction produced is proportional to the percentage elongation as well as to the concentration of the gelatin. He also determined the relation between mechanical stress and produced strain.

Leick's results are given in the following table:

Concentration C	Modulus of elasticity E	$\frac{E}{C^2}$	$\frac{E}{C}$
	Hard gelatin		
10.0	242	2.4	24.2
18.6	978	2.8	52.6
30.0	1545	1.7	51.0
32.0	2157	2.1	67.0
45.0	2944	1.5	65.0
	Soft gelatin		
12.7	323	2.0	25.5
17.5	620	2.0	35.5
19.0	578	1.6	30.5
20.8	619	1.4	29.8
28.3	1000	1.2	35.3
31.7	1189	1.2	37.8
45.6	2327	1.1	51.0

The first three columns were copied from Leick's paper, while the last column was added by the writer. The table shows that Young's modulus of elasticity of gelatin for stretching increases with the concentration, just as double refraction does. But while double refraction increases in direct proportion to the concentration of gelatin, the modulus of elasticity increases, according to Leick's opinion, as the square of concentration, as shown in the third column. But a close examination of the last column shows that with the exception of the cases of 10 per cent for one kind of gelatin and the 45.6 per cent for the other kind, the value of E/C is approximately constant for each kind of gelatin, just as in the case of double refraction. Hence, it appears from Leick's data that the double refraction produced in gelatin by mechanical stress is proportional both to the stress applied and to the dimensional change in the gelatin, *i.e.*, to the produced strain. Leick studied also the effect of addition of salts on the double refraction as well as on the modulus of elasticity of gelatin during stretching. This will be discussed later.

Double Refraction and Swelling of Gelatin

An extensive study of the double refraction which appears in gelatin during swelling was made by Quincke (12). His results may be summarized as follows:

When a solution of gelatin is allowed to set and care is taken to prevent evaporation and drying the formed gel shows no trace of double refraction. If the gel is now placed in a solution in which it swells it becomes doubly refractive. The amount of double refraction is proportional to the *rate* of swelling and it disappears completely when the gel stops swelling. Gels in the form of cylinders or spheres on swelling show the same kind of double refraction as sections of a positive spherulite; but when allowed to shrink in alcohol or glycerin they show the double refraction of a negative spherulite. Gels in the form of prisms when allowed to swell behave with respect to double refraction as if the outside layers of the gel were under tension, while the middle of the block shows negative double refraction, as if it were compressed.

The double refraction in all swollen gels lasts only a short while, from several hours to a few days depending on the size and shape of the block, and then disappears gradually.

Quincke explains this gradual disappearance of double refraction in gels when swollen as a gradual equalization between the compressive stresses in the middle layers of the gel and the tensile stresses in the outer layers. He sees in this phenomenon a proof of his theory of "Shaumstruktur" of gels.

Attempts were made to explain the gradual disappearance of double refraction in a swollen gel by the "relaxation theory" of Maxwell (13), according to which the elastic stresses in a viscous body tend to disappear, so that if the body is left to itself it gradually loses any internal stress, and the pressures are finally distributed as in a fluid at rest. This theory may hold for very dilute gels, but does not apply to concentrated ones at a temperature below 15°C., as observed by Hatschek (14).

It will be shown later that the real reason why double refraction gradually disappears in a swollen gel lies in the tendency of a gel to swell equally in its three dimensions, no matter what the original shape of the block was. Double refraction exists in a swelling gel as long as there is a difference in the amount of swelling in the three dimensions of a block of gel. This is especially true of a thin strip of gelatin. The rate of swelling is most rapid on the flat surface of the strip, and at first the percentage increase in the dimension perpendicular to this surface is much greater than in the other two directions. Hence when observation is made through the narrow edge of the strip very striking double refraction may be observed. But gradually the other dimensions of the strip begin to increase until the percentage change is the same in all of them. As a result of this there is a gradual disappearance of double refraction. If it were possible by any means to control the swelling so as to make the gel swell in one direction only and prevent the swelling from spreading in the direction of the other two dimensions of the gel, the double refraction should then persist as long as the gel is swollen. After various trials the writer found that this could be accomplished most conveniently by casting gelatin in rectangular glass frames or on glass slides. In this way the gelatin swells only in one direction. The double refraction which takes place in the gel persists indefinitely as long as the gel is swollen, and, as will be shown later, is proportional to the swelling.

Technique and Apparatus

All the measurements were done in a cold room kept at 6°C. ± 1°C. Electrolyte-free gelatin was prepared from Cooper's unbleached powdered gelatin by the modified (15) Loeb method. A 2 per cent solution in distilled water became milky white when allowed to stand in a cold room. The pH of the solution was 4.95 and this was taken as the isoelectric point of this stock of gelatin.

Various solutions were made up by dissolving definite weights of air dried gelatin in weighed amounts of water. Corrections were made for the moisture content of the gelatin which happened to be 15 per cent. The concentrations of the gelatin were often checked by drying weighed samples for 24 hours at 100° to 110°C. All weights of gelatin in this paper refer to gelatin dried in this way. The gels were prepared by heating the mixtures of gelatin and water to 50°C., were kept at this temperature until the gelatin grains were completely dissolved, and then put into forms and allowed to set for 20 to 24 hours in the cold room. Two forms of blocks of gelatin were employed in this work. Most of the experiments were done on gels cast in glass frames, but a number of experiments were done also on gels cast on glass slides.

Gels in Glass Frames.—The frames were made from narrow strips of glass about 3 mm. wide cut from microscopic slides 1 mm. thick. The strips were bent into rectangular frames 20 mm. × 10 mm. by means of the blast flame and sealed. Examination with the polarizing microscope showed strain in the frame only at the corners where the bending and sealing had taken place, while in the middle of the longer sides no trace of strain could be detected. The sharp edges of the frame were ground and the frames were marked and numbered. Two lines about 2 mm. apart were drawn with glass ink in the middle of both long sides of each frame. The inner width of the frames at the middle were measured to 0.01 mm. by means of a Zeiss measuring microscope CG. As the sides were seldom absolutely parallel, it was necessary to measure the width at the top and bottom of the frame and use the average of both measurements. The two widths never varied more than 0.2 mm.

The exact weight of the frames was also determined. The frames were placed on slides, filled with liquid gelatin and covered with thin slides. These were then put in rubber stoppered tubes containing some water so as to prevent any possible drying of the gelatin and allowed to remain in the cold room for 20 to 24 hours. The frames containing the gel were afterwards removed from the slides by means of a razor blade, cleaned of any adhering gelatin on the outside walls, weighed, and put into flasks containing various solutions. Examination of the gel in the frame at this stage showed very little if any double refraction. The gels in the frames were examined for double refraction at various lengths of time, usually after about 5, 10 and 20 days.[4] The beam of light from the polarizing Nicol was made to pass through the full width of the frame and the microscope was focussed on the space in the side of the frame between the two marked lines. Thus observations were always made through the same depth and the same location of the gel. The measurements of double refraction were made on the gels while immersed in the solutions in which they were allowed to swell.

At the same time the amount of swelling was determined by drying the frames carefully with filter paper and weighing.

Gels on Glass Slides.—Rectangular glass discs 25 mm. × 15 mm. were cut from microscopic slides, numbered and weighed. These were placed on regular slides supported by small strips of glass about 1 mm. thick, thus leaving an equidistant space of 1 mm. height between the small and larger slides. Liquid gelatin was then allowed to flow into the space between the slides by means of a pipette. The gelatin filled up the space readily, assisted by surface tension.

The slides were then put away in stoppered moist tubes to set for 20 to 24 hours. The gelatin was afterwards carefully removed from the larger slide by means of a razor blade, but allowed to adhere to the smaller slide. A rectangular steel die of 20 mm. × 10 mm. opening was pressed against the gelatin on the small slide thus enabling us to obtain a very uniform rectangular disc of gelatin of definite dimensions and parallel edges on the small slide. The exact length and width of the gel discs on the slides were measured to 0.01 mm. by means of the measuring microscope. It was found very convenient to direct a fine but strong horizontal beam of light, of the kind used in ultramicroscopy, on the gelatin during these measurements so as to produce a Tyndall cone in the gel. This facilitated enormously the focussing on the edges of the disc. The dimensions were determined both at the exposed surface of the gel disc as well as at the surface in contact with the glass slide. The gels were then weighed and put into the swelling medium as in the case of the gels in frames. The optical observations in this case were made through the middle of the long edge of the disc of gelatin.

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[4] It was found necessary to put a crystal of thymol in every solution in order to prevent mould growth on the gelatin.

The two kinds of blocks of gel when placed in water swell differently. The gels in the frames swell in one direction only, namely in thickness, so that the percentage change in total volume of the gel is the same as the percentage change in thickness. The gels which are cast on slides swell largely in thickness, but at the same time the other two dimensions change slightly too, the gel finally appearing as a thin frustum of a rectangular pyramid, with the smaller base adhering to the glass. In the case of the gels cast in the glass frames it is enough to weigh the gel from time to time, in order to be able to determine the changes in its linear dimensions; while in the case of the gels cast on glass slides it is necessary to measure actually the changes in the three dimensions of the gel. As it will be seen later the measured double refraction per unit change in linear dimension is identical in both forms of gels.

The apparatus for measuring double refraction consisted of a Leitz Babinet compensator fitted into an ordinary microscope which was provided with a polarizing Nicol in the substage, and with an analyzer over the eyepiece of the compensator. The microscope was held in horizontal position. A rectangular cell 3 cm. long, 2 cm. wide and 2 cm. high, made of plane optical non-strained glass was fastened by clips to the stage of the microscope and could be moved into various positions in the vertical plane by means of micrometer screws. The gels on the slides or in the frames were placed in the cell in a flat position and covered with the solution in which they had been allowed to swell. The cell was then moved on the stage into such a position that the microscope could be focussed on the middle point of the front edge of the gel. The beam of light was thus made to pass through the full width of the gelatin disc.

A Cooper-Hewitt mercury quartz lamp, the Lab-Arc, was used as a source of illumination. It was provided with two glass filters made by the Corning Glass

Works, a Didymium filter and a G 34-y filter, which allowed only the mercury 5461 Å.U. green light to pass through. This was checked by means of a spectroscope.

The Babinet compensator as arranged in this work showed a positive displacement of the zero line to the left for double refraction corresponding to stretching of gelatin in a vertical direction and a negative displacement to the right when gelatin was compressed in the same direction.

A phase difference of one wave length of 5.461×10^{-4} mm. corresponded to 9.35 divisions of the scale.

Hence if δ represents the reading of the Babinet compensator in division then the phase difference

$$\Delta p = \frac{\delta}{9.35}$$

Substituting in the formula $\Delta_p = \frac{s}{\lambda_0}(n_2 - n_1)$ (Equation 1) values for Δ_p and λ_0 we get

$$n_2 - n_1 = \frac{\delta}{9.35} \times \frac{5.46 \times 10^{-4}}{d} = 5.84 \times 10^{-5} \frac{\delta}{s}$$

where s is equal to the width of the gelatin disc in millimeters. The value of $n_2 - n_1$ is the double refraction produced by the algebraic sum of the elastic strains in the gel in directions lying in a plane perpendicular to the width, *i.e.*, by changes in the thickness and in the length of the gelatin disc. In the case of gels cast in frames the only possible change is in the thickness of the disc which is the same as the change in the total volume of the disc of gel.

Let W_0 be the weight in gm. of the gel when set
V_0 its volume when set
D_g the density of the gel
D_w the density of the water = 1
W_1 the changed weight of the gel after swelling

When the swelling takes place in a very dilute salt solution we have

$$\frac{\Delta V}{V_0} = \frac{(W_1 - W_0)/D_w}{W_0/D_g} = \frac{\Delta t}{t_0} \qquad \text{(Equation 2)}$$

where $\frac{\Delta V}{V_0}$ and $\frac{\Delta t}{t_0}$ are the fractional changes in volume and thickness.

When concentrated salt solutions are used in the outside solution the fractional change in the volume of the gel is

$$\frac{\Delta V}{V_0} = \frac{(W_1 - W_g)/D_s - (W_0 - W_g)/D_w}{W_0/D_g} \qquad \text{(Equation 3)}$$

where W_g = weight of dry gelatin in the gel and D_s = density of salt solution.

In deriving this formula it was assumed that the concentration of salt in grams per 100 gm. H_2O is the same inside of the gel as outside of it, which is true for high concentrations of salts as found by analysis (16).

The final concentration of gelatin in grams per cubic centimeters of H_2O or salt solution is

$$C = \frac{W_g}{(W_1 - W_g)/D_s} \qquad \text{(Equation 4)}$$

The double refraction per unit change in dimension and per unit concentration for gels cast in frames is

$$\alpha = \frac{n_2 - n_1}{\frac{\Delta V}{V_0} C} \qquad \text{(Equation 5)}$$

which may be termed the optical modulus of elasticity of a gel containing 1 gm. of dry gelatin per cubic centimeter of H_2O or salt solution.

When gelatin is cast on slides it swells not only in thickness but also in length, and the double refraction is a function of the value μ where

$$\mu = \frac{\Delta t}{t_0} - \frac{\Delta l}{l_0}$$

It was found impossible to measure accurately the change in thickness of the gels, but there was no difficulty in measuring the changes in length and width. The method employed to determine the change in thickness was to determine the fractional change in volume $\frac{\Delta V}{V_0}$ in the same way as in the case of the gels in the frame. Exact determinations were also made of $\frac{\Delta l}{l_0}$ and $\frac{\Delta w}{w_0}$, *i.e.*, the fractional changes in length and width.

The value of $\frac{\Delta t}{t_0}$, the fractional change in thickness, was then calculated from the formula

$$\frac{\Delta t}{t}\left(1+\frac{\Delta l}{l}+\frac{\Delta w}{w}+\frac{\Delta l}{l}\times\frac{\Delta w}{w}\right)=\frac{\Delta V}{V}-\left(\frac{\Delta l}{l}+\frac{\Delta w}{w}+\frac{\Delta l}{l}\times\frac{\Delta w}{w}\right)$$

When $\frac{\Delta l}{l}$ and $\frac{\Delta w}{w}$ are very small fractions of a unit then the formula becomes

$$\frac{\Delta t}{t}=\frac{\Delta V}{V}-\left(\frac{\Delta l}{l}+\frac{\Delta w}{w}\right)$$

It has been found necessary for accuracy in most of the experiments here to employ the complete formula. The optical modulus of elasticity in the case of the gels on slides is then

$$\alpha=\frac{n_1-n_2}{\mu C} \qquad \text{(Equation 6)}$$

Density Measurements

The density of various concentrations of gels at 6°C. is given in the following table:

Concentration in gm. gelatin per 100 cc. H_2O......	5	6	8	10	12	14	16	18	20
Density of gel after 24 hrs. setting.....	1.019	1.022	1.028	1.033	1.039	1.045	1.051	1.056	1.060

The density of the various gels was determined by weighing discs of gels first in air and then in kerosene in the cold room. The discs of about 0.5 gm. weight were suspended from the beam of a balance by means of a blackened, fine platinum wire.

The density of the various salt and acid solutions used for the swelling experiments was determined in the cold room by means of a Westphal balance.

Precision and Errors

The weight and hence the volume of the gels can be determined with great precision. But there is always a possible error due to the difficulty of removing the last trace of the outside liquid by means of filter paper. Checks have shown that the values for V_1 and V_0 are correct to about 0.5 per cent. The percentage error in the double refraction measurements depends on the magnitude of the reading and is about 0.5 per cent for a magnitude of $(n_2 - n_1)$ of 5×10^{-5} and 5 per cent for $(n_2 - n_1)$ equal to 1×10^{-5}.

The length and width measurements have a percentage error of not more than 0.5 per cent. The probable percentage error in the final calculated value of the optical modulus of elasticity depends on the probable errors in the above mentioned measurements.

From the relation (Equation 5)

$$\alpha = \frac{n_2 - n_1}{\frac{V_0}{V_0} C} = \frac{(n_2 - n_1) V_0}{(V_1 - V_0) C}$$

it follows that the probable percentage error in α equals

$$p\alpha = \sqrt{p^2{(n_2-n_1)} + p^2 V_0 + p^2 C + p^2 (V_1 - V_0)}$$

where p equals the probable percentage error in the various factors designated by the subscripts.

The probable percentage error in C is of the same magnitude as in V_0 or V_1.

An expression for the relation between the probable percentage errors in V_1 and V_0 and in their difference, *i.e.*, in $(V_1 - V_0)$ can be found as follows:

The probable *actual* error E in $(V_1 - V_0)$ due to errors e_1 and e_0 in V_1 and V_0 is

$$E = \sqrt{e_1^2 + e_0^2}$$

But

$$E = \frac{(V_1 - V_0) P}{100}, \quad e_1 = \frac{V_1 p_1}{100}, \text{ and } \quad e_2 = \frac{V_0 p_0}{100}$$

where P, p_1 and p_0 are the probable *percentage* errors in $(V_1 - V_0)$, V_1 and V_0. Hence

$$(V_1 - V_0) P = \sqrt{V_1^2 p_1^2 + V_0^2 p_0^2}$$

Dividing through by $V_1 - V_0$ we get

$$P = \sqrt{\left(\frac{V_1}{V_1 - V_0}\right)^2 p_1^2 + \left(\frac{V_0}{V_1 - V_0}\right)^2 p_0^2}$$

This expression shows that the probable percentage error in $V_1 - V_0$ increases enormously as $V_1 - V_0$ approaches zero.

Thus for small magnitudes of swelling both the errors in the double refraction and in the value of $\frac{\Delta V}{V_0}$ contribute to a possible enormous error in the calculated optical modulus of elasticity.

Swelling of Thin Unsupported Discs of Gelatin

Thin rectangular discs of isoelectric gelatin were cast on glass slides and allowed to set in a cold room for 24 hours. The gels were afterward removed from the glass slides, weighed, measured, and put into 100 cc. $\frac{\text{M}}{1000}$ acetate buffer pH 4.95.

TABLE I

Rate of Change in the Three Dimensions of a Thin Rectangular Disc of Isoelectric Gelatin on Swelling in $\frac{\text{M}}{1000}$ *Acetate Buffer pH 4.95. Concentration: 20 Gm. of Gelatin in 100 Cc. Solution. Weight 0.311 Gm., Length 21.25, Width 9.45 Mm.*

Time in hrs.	1.25	2.25	3.75	6.0	24
Percentage increase in volume	10.30	16.00	20.10	23.40	27.50
" " " length	2.55	4.55	5.70	6.50	8.00
" " " width	1.90	5.45	5.60	6.40	7.95
" " " thickness (calculated)	5.70	5.30	7.50	9.00	9.20

Table I shows the rate of change in the various dimensions of the gelatin. The gel changed most rapidly in thickness, which in about 1 hour gained almost 60 per cent of its total increase while the other two dimensions gained in the same time only about 25 per cent of the final gain. But as the swelling progressed the rate of swelling in thickness diminished while the length and width continued to increase constantly until finally the percentage gain was the same in all three dimensions.

Table II shows the final percentage change in the three dimensions of unsupported discs of gels of various concentrations. In practically all cases there was no difference in the final percentage change of the various dimensions. The double refraction in these gels although very marked at the beginning of swelling disappeared entirely after

24 hours. It is obvious that the reason for its disappearance lies in the fact that the elastic strain became equal in all directions.

TABLE II

Swelling of Unsupported Thin Discs of Gels of Various Concentrations of Isoelectric Gelatin in $\frac{M}{1000}$ *Acetate Buffer pH 4.95 at 6°C. Original Dimensions of Discs about 1.5 × 10 × 20 Mm.*

Concentration in gm. gelatin per 100 gm. H_2O	5	6	8	10	12	14	16	18	20
After 3 days in buffer solution									
Percentage change in length....	−3.9	−4.7	−2.4	0	+1.8	+3.0	+5.0	+5.6	+6.9
" " " width.....	−5.3	−5.3	−2.5	−.5	+1.4	+2.8	+5.2	+6.6	+7.6
" " " thickness.	−8.8	−4.4	−2.8	−1.2	+.6	+4.2	+3.7	+6.0	+7.2

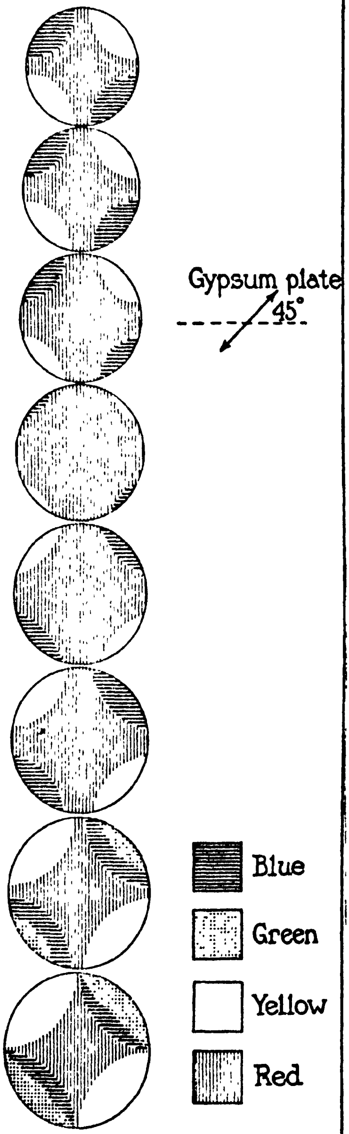
Unsupported gels are therefore entirely unsuitable for a quantitative study of the relation between double refraction and swelling; hence all the following quantitative experiments were made with gels supported on glass slides or cast in glass frames.

Effect of Concentration

It has been shown by the writer in his paper on syneresis (17) that when solid blocks of gelatin are placed in cold dilute buffer solution of the same pH as that of the isoelectric point of the gelatin, only those of a gelatin content of more than 10 per cent swell, while those of a lower gelatin content not only do not swell but actually lose water. This was also shown here in Table II. In working out the theory of this phenomenon the writer made the assumption that at setting all the gels no matter of what concentration are under no elastic stress, but when placed in dilute buffer solution the gels of a concentration of higher than 10 per cent are under tensile stresses due to swelling, while below 10 per cent the gels are under compressive stress resulting from shrinking. Both forms of internal stresses act against the forces which cause swelling on the one hand and shrinking on the other hand, namely the osmotic force due to the soluble ingredient of gelatin and the strain in the micells of the gelatin.

With the object in view of finding out whether double refraction

Concentration of gelatin in gm. per 100 gm. H_2O	Percentage increase in weight	Appearance of gels in polarizing microscope over gypsum plate R_1
5	-14.4	
6	-12.3	
8	- 6.6	
10	-1.3	
12	+ 4.1	
14	+ 6.6	
18	+15.6	
20	+18.0	



Text-Fig. 1. Interference colors in cylinders of gels of various concentrations.

would show the difference in the form of stress in blocks of gelatin of various concentrations the following series of experiments were made.

1. Observation of Interference Colors in Cylinders of Various Gelatin Content.—Gelatin solutions of various concentrations were allowed to set for 24 hours in short glass tubes of about 8 mm. diameter, stoppered at both ends. The gels were removed next day by means of a cork borer and cut into short cylinders of about 10 mm. high and 5 mm. in diameter. These were weighed and put into 100 cc. $\frac{M}{1000}$ acetate buffer pH 4.95.

TABLE III

Swelling and Double Refraction of Blocks of Various Concentrations of Gelatin Cast in Frames. Outside Solution $\frac{M}{1000}$ *Acetate Buffer pH 4.95*

Gm. gelatin per 100 gm. H_2O	12	14	16	18	20
After 20 Days:					
$100\frac{\Delta V}{V_0}$	5.00	8.84	13.16	17.33	20.30
$100\,C$	11.40	12.75	13.90	15.00	16.15
$(n_2 - n_1) \times 10^5$	2.29	4.20	6.75	10.22	11.54
$\frac{n_2 - n_1}{\frac{\Delta V}{V_0} C} \times 10^3$	4.01	3.72	3.68	3.93	3.53

Text-fig. 1 shows schematically the colors in the gels after 36 hours, when observed through the top of the cylinder between crossed Nicols in combination with a gypsum test plate R_1. Ordinary light was used as a source of illumination.

The difference in the position of the blue and yellow colors in the gels of a higher or lower concentration than 10 per cent is very striking. In the 10 per cent gel there is very little blue or yellow color except red due to the gypsum plate.

2. The Quantitative Relation Between Double Refraction and Swelling or Shrinking in Various Concentrations of Gelatin.—Tables III and IV give the results of observations on gels cast on slides as well as on gels cast in frames. The frames are not suitable for gels which shrink when placed in water, since the gels become detached from the glass.

TABLE IV

Swelling and Double Refraction of Blocks of Various Concentrations of Gelatin Cast on Slides

V = volume, l = length, w = width, t = thickness

Gm. gelatin per 100 gm. H_2O	5	6	8	12	14	16	18
After 24 Days:							
$100\frac{\Delta V}{V_0}$	−21.3	−19.8	−11.0	+4.87	+10.05	+16.10	+22.3
$100\frac{\Delta l}{l_0}$	−2.70	−1.90	−1.04	+.24	+.64	+.85	+1.39
$100\frac{\Delta w}{w_0}$	−4.67	−5.10	−2.74	+.10	+1.27	+2.54	+2.96
$100\frac{\Delta t}{t_0}$	−13.75	−11.86	−6.90	+4.65	+8.55	+12.30	+17.15
$100\mu = 100\frac{\Delta t}{t_0} - \frac{\Delta l}{l_0}$	−11.05	−9.96	−5.86	4.41	+7.91	+11.45	+15.76
$100\,C$	6.40	7.54	9.00	11.40	12.53	13.57	14.40
$(n_2 - n_1) \times 10^6$	−2.54	−2.98	−2.35	+1.79	+4.03	+6.08	+9.00
$\frac{n_2 - n_1}{\mu C} \times 10^3$	3.59	3.94	4.45	3.56	4.06	3.92	3.96

Some of the gels were removed from the glass and left in the $\frac{M}{1000}$ acetate buffer for 8 days longer

Final Measurements:							
$100\frac{\Delta V}{V_0}$				+5.1		+20.9	+27.2
$100\frac{\Delta l}{l_0}$				+1.00		+4.27	+5.36
$100\frac{\Delta w}{w_0}$				+1.05		+5.08	6.74
$100\frac{\Delta t}{t_0}$				+2.9		+10.3	+13.1
100μ				+1.9		+6.0	+7.7
$100\,C$				11.4		13.0	13.7
$(n_2 - n_1) \times 10^6$				+.92		+3.24	+4.09
$\frac{n_2 - n_1}{\mu C} \times 10^3$				4.25		4.15	3.88

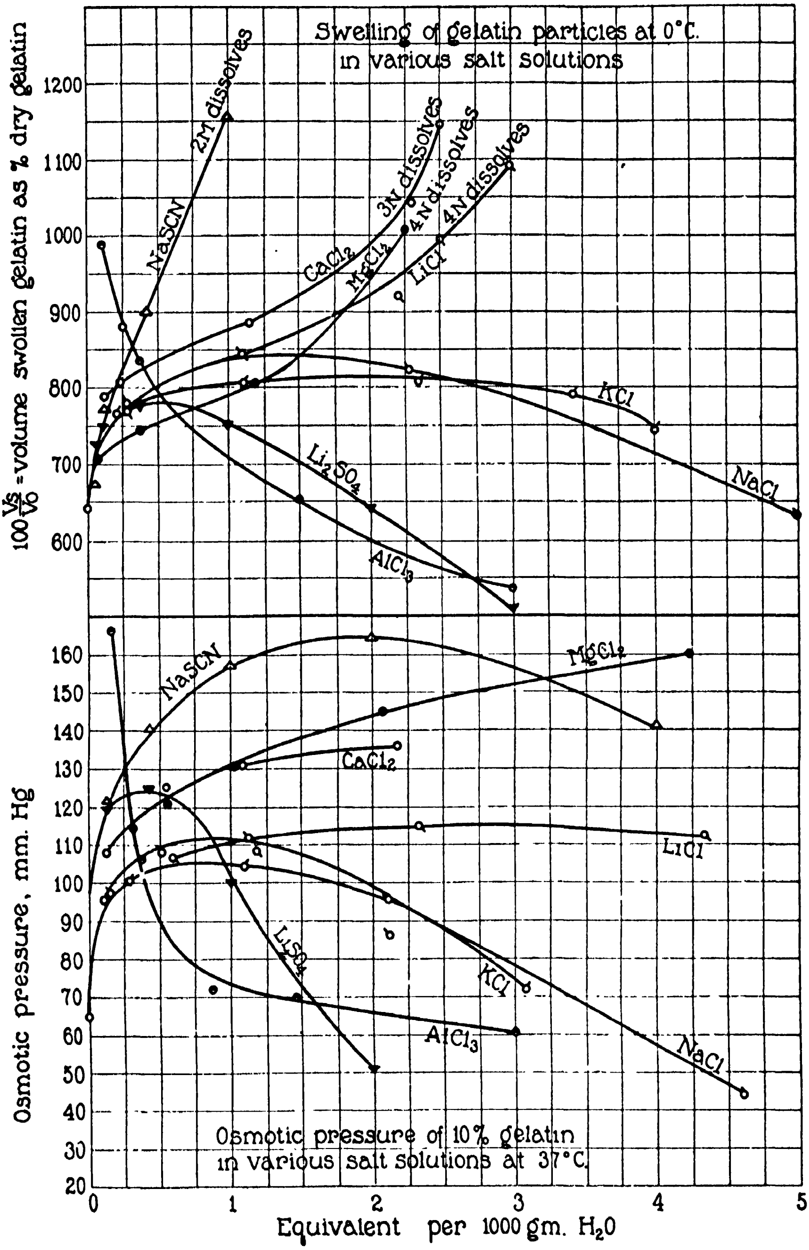
The tables show that when gels containing various amounts of dry gelatin per 100 cc. H_2O are placed in cold $\frac{M}{1000}$ acetate buffer of the same pH as that of the isoelectric point of the gelatin, those of less than 10 per cent shrink and the double refraction is negative, as if the gels were compressed, while those gels of above 10 per cent swell and the double refraction is positive, showing that the gels are under tension. At a concentration of 10 per cent the gel does not change and there is no double refraction. The double refraction generally increases with the increase in concentration.

Simple calculation shows that the double refraction is proportional both to the change in the thickness of the gels (or to the difference between the percentage changes in thickness and in length) and to the concentration of the gel. The optical modulus of elasticity of the gels, namely the double refraction per unit change in dimension and per unit concentration, is the same both for swelling and shrinking, giving a value of about 4.0×10^{-3} which is very close to the value obtained by Leick for the double refraction in gelatin produced by stretching.

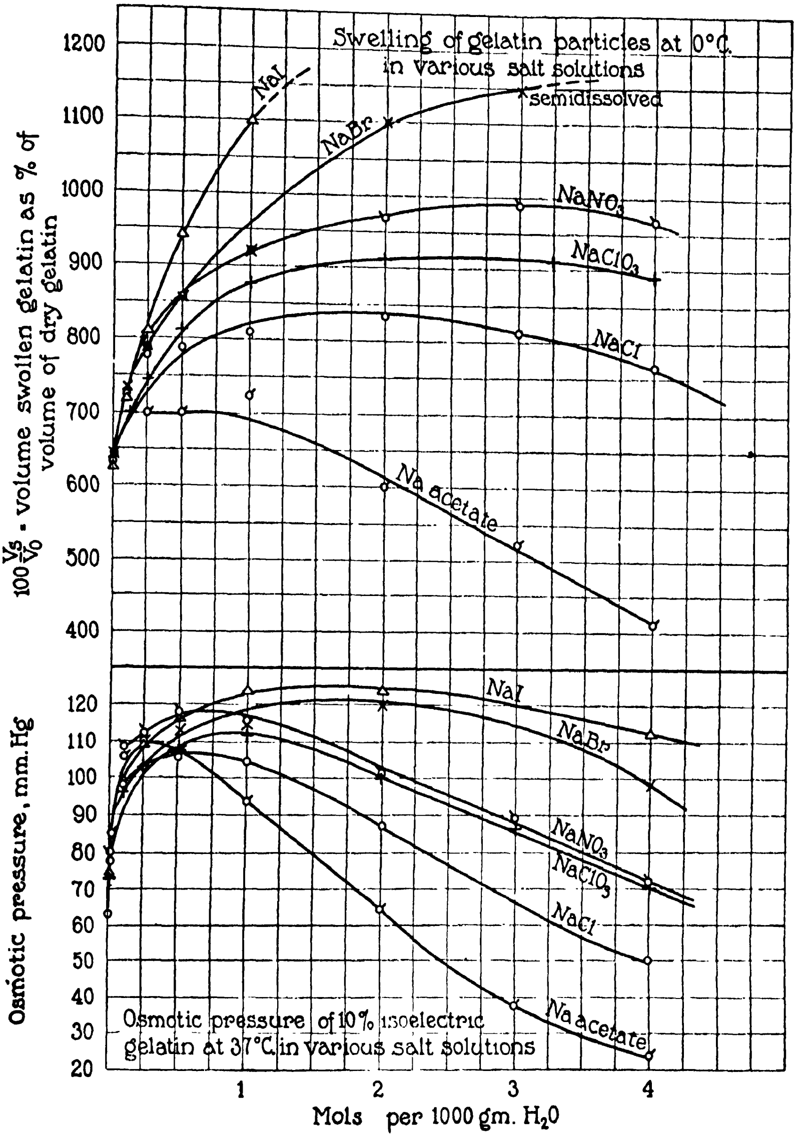
This value for double refraction per unit change in dimension and per unit concentration in the gels does not depend on the form of glass support. It is the same both for gels in glass frames and for gels cast on slides, although the mode of swelling is quite different in the two forms of gels. It does not change even when the gels are removed from the glass, as shown in the second part of Table IV. The length and width of the gels increase on removal from the glass, while the thickness diminishes. The double refraction is also reduced. But the ratio of the double refraction to the change in dimensions and the concentration remains the same as when the gels were still attached to the glass.

Effect of Salts on Swelling and Double Refraction of Isoelectric Gelatin

The effect of salts on swelling of gelatin in acids or alkalies has been studied extensively by Loeb and the writer (18). Northrop and the writer (6) have also studied in detail the effect of salts on the swelling of isoelectric gelatin in the absence of either acid or alkali. The effect of salts is quite different in the two cases. Salts always depress the

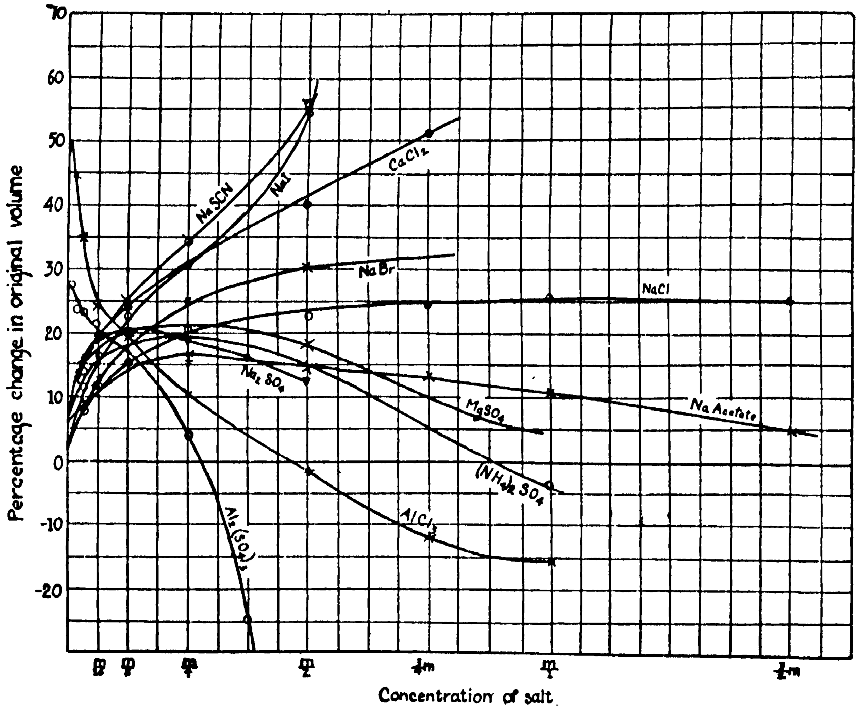


TEXT-FIG. 2. Comparison of osmotic pressure and swelling of gelatin in various salt solutions. (Reprinted from *J. Gen. Physiol.*, 1926, **8**, 322.)



TEXT-FIG. 3. Comparison of osmotic pressure and swelling of gelatin in various salt solutions. (Reprinted from *J. Gen. Physiol.*, 1926, 8, 323.)

swelling produced by acid or alkali, and the amount of depression depends on the valency of the ions, while in the absence of acid or alkali, salts generally increase the swelling of isoelectric gelatin. The peculiar effect of salts on swelling of isoelectric gelatin was found to be similar to the effect of salts on the osmotic pressure of isoelectric gelatin solutions. It was found, namely, that salts generally increase the osmotic pressure of gelatin solutions and the curves (Text-figs. 2 and 3) both for swelling and osmotic pressure run parallel throughout a wide range of concentrations of salts. This suggests that the effect of salts on swelling is due to an increase in the osmotic pressure of the gel caused either by increase in the concentration of the osmotically active soluble component of the gel (2) or by an unequal distribution of ions through the formation of complex ions with the gelatin (16).



TEXT-FIG. 4. Swelling of 10 per cent gels of isoelectric gelatin in various salt solutions at 6°C.

On the other hand, it was found that in the case of such salts as

NaSCN, NaI, NaBr, LiCl and $CaCl_2$ the parallelism between the osmotic pressure and swelling curves holds only for low concentrations, while at concentrations above $\frac{M}{2}$ the osmotic pressure curves begin to drop with increase in concentration of salt, while the swelling curves continue to rise. These salts apparently have a double effect on swelling, first, they increase the osmotic pressure of the gel, and second, they decrease the elasticity of the gel and thus reduce the resistance of the gel to swelling.

A detailed study was hence undertaken on the effect of salts on the double refraction in gels during swelling in order to determine directly how the elasticity of the gels is affected by various salts.

Some studies on the effect of salts on the elasticity of gels during mechanical deformation have been made by several investigators. Fraas (19) found that NaCl makes gelatin set slower and decreases the tenacity of the gel. Leick (11) found that chlorides lower the specific double refraction and the elastic modulus of stretching of gels while sodium sulfate produces no change in the elastic properties of gelatin. Leick employed rather high concentrations of salts, the minimum being about half molar. Sheppard and his collaborators (20) studied the effect of potassium alum as well as of acid and alkali on the modulus of rigidity of gels.

In the present study of the effect of salts on the double refraction of gelatin during swelling advantage was taken of the fact that when a 10 per cent gel is placed in distilled water, which had been adjusted by means of acid to the pH of the isoelectric point of gelatin, the gel does not swell. Addition of salt causes swelling and double refraction. The relation between the amount of swelling and the double refraction produced will thus indicate the elastic condition of the gel due to the salt alone.

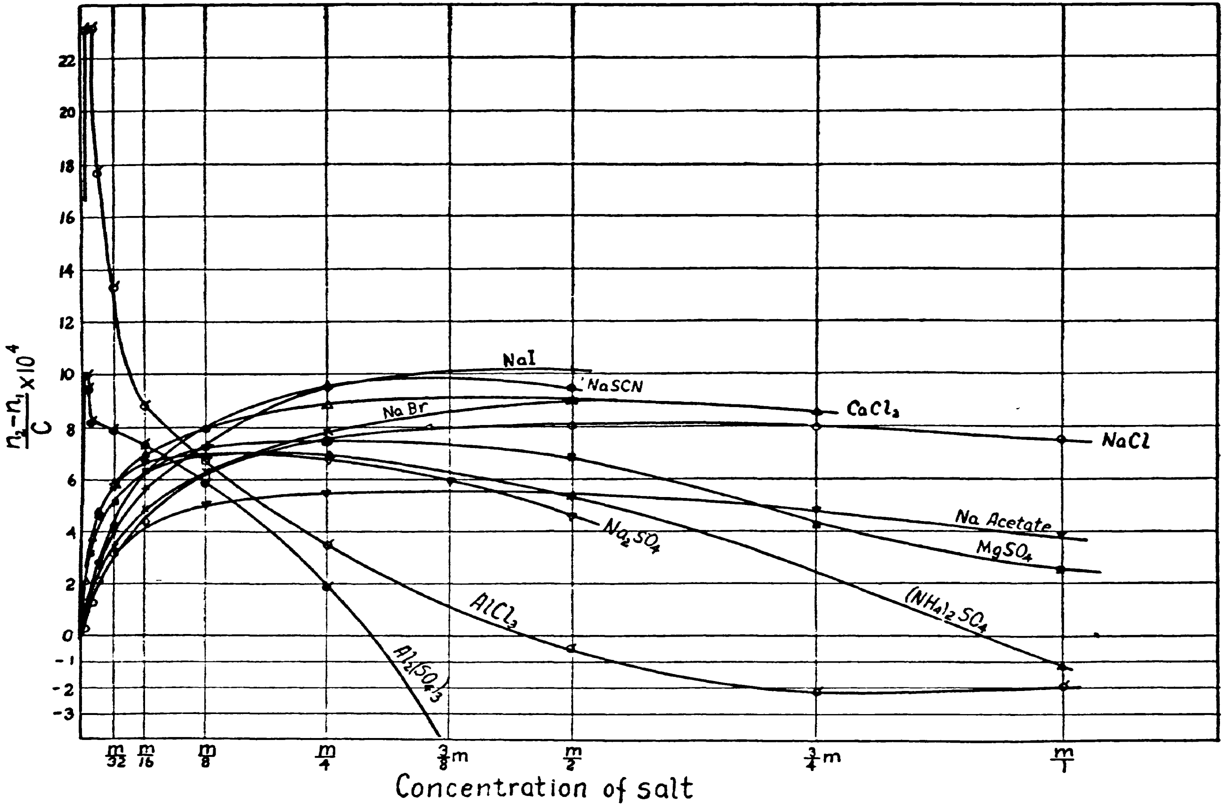
Salts generally change the pH of gelatin, which may affect the amount of swelling. But, as will be shown later, a variation in the pH of gelatin between 3.0 and 9.0 has no influence on the elastic properties of a 10 per cent gel. Hence, no extra precautions were taken to control the pH of the salt solutions used, except in the case of sodium acetate where acetic acid was added to bring the solutions to pH 4.95. In all other cases the weighed dry salt was dissolved in H_2O acidified to pH 4.95 with acid of the same anion as the salt, whenever possible, and the dilutions were made with this solution.

TABLE V

Swelling and Double Refraction of 10 Per Cent Gels in Various Salt Solutions at 6°C. Gels Cast in Glass Frames. Weight of Gels about 0.8 Gm. Measured after 19 to 24 Days

Concentration of salt solution.....	2 M	$\frac{3}{2}$ M	$\frac{M}{1}$	$\frac{3}{4}$ M	$\frac{M}{2}$	$\frac{3}{8}$ M	$\frac{M}{4}$	$\frac{M}{8}$	$\frac{M}{16}$	$\frac{M}{32}$	$\frac{M}{64}$	$\frac{M}{128}$	$\frac{M}{256}$	$\frac{M}{512}$	$\frac{M}{1024}$	$\frac{M}{2048}$
NaCl:																
$\frac{\Delta V}{V_0} \times 10^2$.............	25.6	25.1	25.5	24.4	22.8		20.4	15.4	11.8	7.86	5.47	2.89	1.55	−.24		
$(n_2 - n_1) \times 10^5$.......	5.12	6.00	5.88	6.34	6.50		6.18	5.22	3.89	2.93	2.01	1.21	.29	−.36		
NaBr:																
$\frac{\Delta V}{V_0} \times 10^2$.............					30.3		25.0	18.0	13.0	8.83	5.03	2.20	.34	−1.0		
$(n_2 - n_1) \times 10^5$.......					6.80		6.16	5.28	4.27	3.13	1.92	.84	0	−.13		
NaI:																
$\frac{\Delta V}{V_0} \times 10^2$.............					54.2		30.9	22.7	16.3	11.7	6.34	3.72	2.22	.67		
$(n_2 - n_1) \times 10^5$.......					6.42		7.18	5.87	4.82	3.55	2.42	1.31	.40	.09		
NaSCN:																
$\frac{\Delta V}{V_0} \times 10^2$.............					55.8		34.3	24.8	17.9	11.2	6.30	2.50				
$(n_2 - n_1) \times 10^5$.......					5.96		6.86	6.20	5.37	3.74	2.63	1.02				
Na Acetate pH 4.95 (+ Acetic Acid):																
$\frac{\Delta V}{V_0} \times 10^2$.............		5.0	10.4	13.3	14.7	16.3	14.0	11.7	9.06	8.68	4.09	2.69	1.01			
$(n_2 - n_1) \times 10^5$.......		1.66	3.45	4.20	4.65	4.65	4.35	3.86	3.10	2.43	1.68	.82	.24			

$CaCl_2$:																
$\frac{\Delta V}{V_0} \times 10^2$				51.7	39.9		30.7	24.2	20.5	16.3	13.8	9.80	7.02	4.62	2.43	1.66
$(n_2 - n_1) \times 10^5$				5.53	6.38		6.61	6.31	5.70	4.89	4.17	3.33	2.56	1.89	.78	.48
$AlCl_3$:																
$\frac{\Delta V}{V_0} \times 10^2$			−15.3	−12.0	−1.54		+10.6	19.6	24.0	35.9	44.4	53.0		54.7	42.8	27.3
$(n_2 - n_1) \times 10^5$			−2.32	−2.43	−.46		+3.16	5.56	7.06	9.52	11.1	14.8		14.7	12.3	8.72
$Al_2(SO_4)_3$:																
$\frac{\Delta V}{V_0} \times 10^2$						−24.0	+3.80	17.3	22.0	23.0	23.4	26.8	26.0	26.9	26.3	22.6
$(n_2 - n_1) \times 10^5$						−8.3	+1.51	4.88	5.72	6.31		7.33	7.68	7.86	7.91	7.60
Na_2SO_4:																
$\frac{\Delta V}{V_0} \times 10^2$					12.1	15.9	18.3	20.4	17.7	16.1	12.6	8.70	5.56	4.29	.45	
$(n_2 - n_1) \times 10^5$					4.08	5.09	5.64	5.90	5.61	4.94	4.04	3.07	1.87	.85	.11	
$(NH_4)_2SO_4$:																
$\frac{\Delta V}{V_0} \times 10^2$			−3.72		+14.5		19.6	18.7	19.3	14.9	10.0	7.80				
$(n_2 - n_1) \times 10^5$			−1.22		+4.58		5.74	5.76	5.34	4.89	3.84	2.87				
$MgSO_4$:																
$\frac{\Delta V}{V_0} \times 10^2$			5.19	10.1	18.7		21.1	20.2	18.0	13.9	11.3	8.58	5.46			
$(n_2 - n_1) \times 10^5$			2.44	3.75	5.71		6.13	5.94	5.28	4.42	3.98	2.82	2.08			



TEXT-FIG. 5. Double refraction in 10 per cent gels of isoelectric gelatin produced by swelling in various salt lutions at 6°C. The observed double refraction was corrected for changes in the concentration of gelatin during gelling

Table V gives a summary of the results obtained on the swelling and double refraction of 10 per cent isoelectric gels when put into various salt solutions. Experiments were performed both with gels in frames and gels on slides. The results are practically identical in both cases, except that for small values of swelling the results are subject to greater error in the case of gels on slides, where in addition to the weight the length and width of the gels also have to be measured, than in the gels in frames where only the weight of the gel is determined. The values for swelling and double refraction given in this table are those of gels cast in frames, and were taken after the gels had been allowed to remain in the salt solutions for about 20 days.

The plotted curves for swelling (Text-fig. 4) are practically identical with those observed by Northrop and the writer on the swelling of dry isoelectric gelatin granules, except for the curves for aluminum salts which show that in concentrations above $\frac{M}{4}$ both aluminum chloride and aluminum sulfate instead of causing swelling cause shrinking in 10 per cent gels at 6°C. This is also true in the case of $\frac{M}{1}$ ammonium sulfate.

The plotted curves (Text-fig. 5) for double refraction (corrected for the changed concentration[5] of the gelatin) run parallel to those of swelling for most of the salts. But in the case of salts which produce high swelling, namely, NaSCN, NaI and NaBr the double refraction curves do not rise with increase in concentration as fast as the swelling curves, which shows that in spite of the high swelling caused by these salts the internal stresses in these gels, as indicated by the double refraction observed, are not much different than in the gels kept in

---

[5] The final concentration of gelatin in the gels in gram per cubic centimeter of salt solution can be calculated from tabulated values of $\frac{\Delta V}{V_0}$ by means of the following equation:

$$C = \frac{1}{10.66\frac{\Delta V}{V_0} + 10.00}$$

which is easily derivable from Equations 3 and 4.

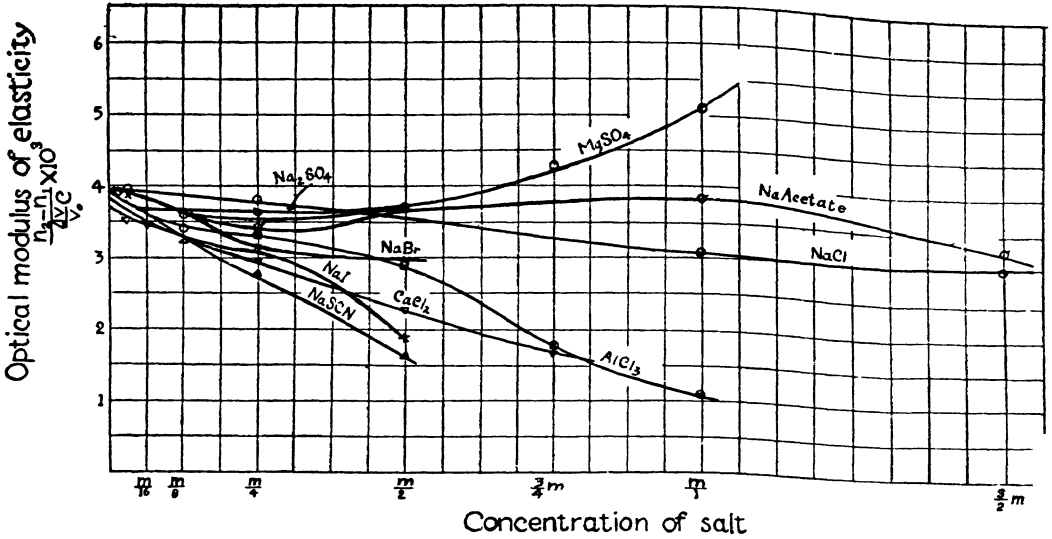
TABLE VI

The Optical Modulus of Elasticity of 10 Per Cent Gels in Various Salt Solutions at 6°C.

$$\frac{n_2 - n_1}{\frac{\Delta V}{V_0} C} \times 10^3$$

Concentration of salt solution	2 M	$\frac{3}{2}$ M	$\frac{M}{1}$	$\frac{3}{4}$ M	$\frac{M}{2}$	$\frac{M}{4}$	$\frac{M}{8}$	$\frac{M}{16}$	$\frac{M}{32}$	$\frac{M}{64}$	$\frac{M}{128}$	$\frac{M}{256}$	$\frac{M}{512}$	$\frac{M}{1024}$	$\frac{M}{2048}$
NaCl:															
10 days	2.68	3.00	3.22		3.61	3.91	3.87	3.58	3.90	3.71	3.76				
24 "	2.54	3.03	2.95		3.53	3.68	3.95	3.69	3.99	3.88	(4.31)				
NaBr:															
14 days					2.92	3.09	3.70	3.87	3.83	3.98	4.09				
22 "					3.02	3.15	3.53	3.77	3.92	4.06	3.94				
NaI:															
13 days					1.96	3.04	3.42	3.46	3.55	4.16	3.73				
19 "					1.86	3.13	3.21	3.49	3.41	4.14	3.60				
Na Acetate:															
6 days		3.12	3.96		3.72	3.42	3.60	3.70	3.72	4.01	3.90				
16 "		3.49	3.67		3.69	3.36	3.56	3.70	3.75	3.89	4.29				
NaSCN:															
8 days					1.96	2.97	3.42	3.69	4.04		4.20				
16 "					1.83	2.82	3.30	3.63	3.85		4.20				
24 "					1.70	2.77	3.17	3.57	3.75		4.15				
Na_2SO_4:															
9 days					3.61	3.57	3.51	3.58	3.66		3.75				
22 "					3.79	3.68	3.52	3.76	3.60	3.63	3.86				

$(NH_4)_2SO_4$:															
7 days			3.64		3.65	3.72	3.87		3.91	4.28	4.11				
15 "			3.23		3.62	3.64	3.83		3.76	4.10	4.05				
22 "			3.17		3.64	3.55	3.70		3.77	4.24	4.00				
$MgSO_4$:															
7 days			5.04	4.26	3.87	3.61	3.68	3.63	3.77	3.66	3.87	4.18			
15 "			5.28	4.34	3.78	3.66	3.57	3.54	3.77	3.89	3.90	3.86			
22 "			4.95	4.13	3.67	3.55	3.57	3.49	3.64	3.96	3.59	4.03			
$CaCl_2$:															
5 days				1.57	2.36	2.99	3.40	3.52	3.59	3.62	3.89	3.65	4.35		
12 "					2.27	3.01	3.31	3.46	3.53	3.59	3.72	3.76	3.98		
21 "				1.66		2.86	3.28	3.39	3.53	3.46	3.75	3.92			
$AlCl_3$:															
5 days			1.35	1.65	2.20	3.55	3.42	3.90	3.75	3.70	4.25		4.35	3.98	4.06
13 "			.90	1.76		3.32	3.46	3.80	3.75	4.20			4.27	3.91	4.06
24 "			1.27	1.75	2.94	3.32	3.42	3.68	3.69	3.68	4.38		4.25	4.19	4.14
$Al_2(SO_4)_3$:															
8 days						4.58	3.19	3.29	3.36	3.41	3.62	3.68	3.82	3.64	4.03
21 "						4.15	3.35	3.20	3.42	3.46	3.46	3.77	3.77	3.85	4.16
30 "						4.38	3.36	3.26	3.41	3.44	3.43	3.69	3.78	3.76	4.08



TEXT-FIG. 6. The effect of salts on the elasticity of 10 per cent gels as calculated from double refraction measurements.

NaCl. These salts apparently lower the resistance of the gel to swelling by decreasing its elasticity.

Table VI and Text-fig. 6 give the values for the optical modulus of elasticity of the gels in the various salt solutions, namely, the value of double refraction per unit change in volume at unit concentration of gelatin, *i.e.*,

$$\frac{n_2 - n_1}{\frac{\Delta V}{V_0} C}$$

where C is the concentration of gelatin in gram per cubic centimeter of H_2O or salt solution at the time of measurement.

The curves show clearly how salts affect the elasticity of gels. In concentrations below $\frac{M}{8}$ most of the salts have practically no effect on the elasticity of gelatin, although they cause swelling. The optical modulus is the same here as the one obtained for swelling of various concentrations of gels in $\frac{M}{1000}$ acetate buffer pH 4.95. In concentrations higher than $\frac{M}{8}$ some salts change the elasticity of the gel, while others do not. Magnesium sulfate and also aluminum sulfate appear to increase the elasticity of the gels. Salts like NaSCN, NaI, $CaCl_2$ and NaBr which bring about rapid increase of swelling with increase in concentration exert a strong diminishing effect on the elasticity of the gels.

This difference in the effect of various salts on the optical modulus of elasticity of gels explains the discrepancy noted before between the osmotic pressure and the swelling curves for gelatin in the presence of salts, namely, that at low concentration the osmotic pressure curves for all salts rise with the concentration of salt, reach a maximum at about $\frac{M}{2}$, and then drop at higher concentrations of salt, while the swelling curves in the case of such salts as NaBr, NaI, NaSCN, $CaCl_2$ and others, continue rising with the increase in concentration. These salts lower considerably the elasticity and hence the resistance of the gels to swelling. The elasticity decreases rapidly with increase in concentration of salt; hence the swelling continues increasing with the concentration until the gels become too soft to stand any swelling pressure and fall apart, or dissolve in very high concentrations of these salts.

Aluminum chloride in high concentrations produces a depression both in osmotic pressure and in swelling of gelatin, so that in solutions of above $\frac{M}{2}$ the gels shrink and lose in volume. The double refraction measurements show that the optical modulus of elasticity is decreased rapidly in concentrations above $\frac{M}{2}$. The resistance to shrinkage of the block is thus decreased with the result that the drop of the curve for swelling is more pronounced than the drop in the osmotic pressure curve.

The effect of salts on the elasticity of gelatin appears mostly in concentrations above $\frac{M}{8}$, while below that the elasticity remains constant. Loeb, in his studies on the effect of salt on swelling of gelatin in the presence of acid or alkali, limited himself mostly to low concentrations of salt, and thus by eliminating the elasticity variable was able to demonstrate clearly the valency rule in the action of salts on the swelling of gelatin caused by acid or alkali.

Swelling and Double Refraction of Gelatin in Acid and Alkali

The double refraction produced in 10 per cent gels when allowed to swell in various concentrations of HCl is shown in Table VII. The gels when placed in solutions of HCl in the concentration range of $\frac{M}{32}$ to $\frac{M}{2048}$ swell to such a high extent that they will not remain attached to glass when the gels are cast in frames or on slides. As seen from the table the optical modulus of elasticity of the gels immersed in the higher concentrations of HCl decreases rapidly both with the increase in concentration of acid used and with time. As a result of this the swelling of the gels in acid keeps on increasing with time and in high concentrations finally dissolves completely. The second rise in the swelling curve of gelatin in acid above $\frac{M}{2}$ is due to the rapid drop in the elasticity of the gel.

TABLE VII

Swelling and Double Refraction of 10 Per Cent Gels in Various Concentrations of HCl at 6°C.

Concentration of HCl	$\frac{M}{1}$	$\frac{M}{2}$	$\frac{M}{4}$	$\frac{M}{8}$	$\frac{M}{16}$		$\frac{M}{4096}$	$\frac{M}{8192}$
Final pH of outside solution							3.45	3.95
After 9 Days:								
$\frac{\Delta V}{V_0} \times 10^2$	53.4	29.8	33.9	47.9	68.2	In $\frac{M}{32}$ – $\frac{M}{2048}$ the gels swell enormously and fall out of the frames	24.0	9.1
$C \times 10^2$	6.36	7.58	7.35	6.61	5.78		7.96	9.11
$(n_2 - n_1) \times 10^5$	2.8	4.96	6.66	9.17	12.2		8.12	3.44
$\frac{n_2 - n_1}{\frac{\Delta V}{V_0} C} \times 10^3$	.82	2.19	2.67	2.89	3.10		4.24	4.19
After 17 Days:								
$\frac{\Delta V}{V_0} \times 10^2$	Enormous swelling, gels too soft to dry and weigh	44.2	43.3	55.6	77.8		28.1	9.90
$C \times 10^2$		6.80	6.86	6.28	5.45		7.69	9.00
$(n_2 - n_1) \times 10^5$		3.99	6.60	9.25	12.40		8.84	3.83
$\frac{n_2 - n_1}{\frac{\Delta V}{V_0} C} \times 10^3$		1.33	2.23	2.64	2.93		4.09	4.30
After 24 Days:								
$\frac{\Delta V}{V_0} \times 10^2$		53.7	51.5	62.4	85.4		30.2	11.1
$C \times 10^2$		6.36	6.46	6.00	5.22		7.56	8.95
$(n_2 - n_1) \times 10^5$		3.56	6.60	9.30	12.45		9.13	4.05
$\frac{n_2 - n_1}{\frac{\Delta V}{V_0} C} \times 10^3$		1.04	1.98	2.48	2.79		4.00	4.06

Table VIII shows the relation between swelling and the double refraction produced in gels placed in $\frac{M}{16}$ NaCl which contains various

TABLE VIII

Swelling and Double Refraction of 10 Per Cent Gels in Various Concentrations of HCl Made Up in $\frac{M}{16}$ NaCl

Concentration of HCl	$\frac{M}{8}$	$\frac{M}{16}$	$\frac{M}{32}$	$\frac{M}{64}$	$\frac{M}{256}$	$\frac{M}{512}$	$\frac{M}{1024}$	$\frac{M}{2048}$	$\frac{M}{4096}$	$\frac{M}{8192}$	$\frac{M}{16384}$	0
Final pH of outside solution		1.3	1.6	1.9	2.7	2.9	3.3	4.0	4.4	4.8	5.0	5.2
After 6 Days:												
$\frac{\Delta V}{V_0} \times 10^2$	36.3	45.7	51.7	61.2	61.8	51.0	36.8	20.3	11.24	10.25	10.66	11.05
$C \times 10^2$	7.21	6.72	6.45	5.95	6.02	6.48	7.17	8.20	8.92	9.00	8.98	8.95
$(n_2 - n_1) \times 10^5$	8.41	10.10	11.26	13.15	13.00	12.20	9.64	6.72	3.97	3.40	3.75	3.70
$\frac{n_2 - n_1}{\frac{\Delta V}{V_0} C} \times 10^3$	3.21	3.29	3.37	3.61	3.50	3.70	3.65	4.05	3.96	3.68	3.92	3.74
After 14 Days:												
$\frac{\Delta V}{V_0} \times 10^2$	44.7	54.1	61.4	Excessive swelling and deformation of gel	73.3	60.4	44.5	24.4	13.60	11.75	12.10	12.7
$C \times 10^2$	6.78	6.34	6.05		5.60	6.08	6.77	7.93	8.72	8.97	8.86	8.8
$(n_2 - n_1) \times 10^5$	8.50	10.30	11.66		13.60	12.60	10.50	7.30	4.64	3.84	4.10	4.2
$\frac{n_2 - n_1}{\frac{\Delta V}{V_0} C} \times 10^3$	2.76	3.00	3.14		3.31	3.43	3.49	3.77	3.91	3.64	3.83	3.8
After 20 Days:												
$\frac{\Delta V}{V_0} \times 10^2$	46.6	55.7	63.5		75.2	62.0	45.9	25.1	13.8	11.9	11.9	12.7
$C \times 10^2$	6.68	6.24	5.96		5.54	5.96	6.70	7.88	8.71	8.88	8.88	8.8
$(n_2 - n_1) \times 10^5$	8.47	10.60	12.0		13.95	13.10	10.60	7.80	4.76	3.87	4.18	4.2
$\frac{n_2 - n_1}{\frac{\Delta V}{V_0} C} \times 10^3$	2.72	3.05	3.17		3.34	3.54	3.44	3.94	3.96	3.66	3.95	3.8

TABLE IX

Swelling and Double Refraction of 10 Per Cent Gels in Various Concentrations of H_2SO_4 at 6°C.

Concentration of H_2SO_4	$\frac{M}{1}$	$\frac{M}{2}$	$\frac{M}{4}$	$\frac{M}{8}$	$\frac{M}{16}$	$\frac{M}{32}$	$\frac{M}{64}$	$\frac{M}{128}$	$\frac{M}{512}$	$\frac{M}{1024}$	$\frac{M}{4096}$	$\frac{M}{8192}$	$\frac{M}{16384}$
Final pH of outside solution						1.5	1.7	2.0	2.8	3.0	3.6	3.8	4.0
After 6 Days:													
$\frac{\Delta V}{V_0} \times 10^2$	35.4	28.8	28.2	31.6	35.0	44.7	52.0	62.0	76.4	70.8	37.4	21.9	9.75
$C \times 10^2$	7.26	7.64	7.70	7.48	7.27	6.78	6.42	6.02	5.51	5.70	7.15	8.10	9.05
$(n_2 - n_1) \times 10^5$	3.86	5.70	6.30	7.41	8.58	9.46	11.60	13.40	14.06	14.35	10.06	7.15	3.62
$\frac{n_2 - n_1}{\frac{\Delta V}{V_0} C} \times 10^3$	1.50	2.59	2.90	3.13	3.37	3.12	3.48	3.59	3.34	3.55	3.77	4.03	4.10
After 16 Days:													
$\frac{\Delta V}{V_0} \times 10^2$	82.2	46.5	37.4	39.1	42.2	52.5	58.5	72.0	88.4	84.4	45.0	26.7	11.6
$C \times 10^2$	5.32	6.68	7.15	7.05	6.89	6.42	6.15	5.65	5.14	5.26	6.76	7.77	8.90
$(n_2 - n_1) \times 10^5$	1.08	4.93	6.36	7.60	8.64	9.60	11.70	13.8	14.50	14.55	11.45	8.36	4.02
$\frac{n_2 - n_1}{\frac{\Delta V}{V_0} C} \times 10^3$	.25	1.59	2.38	2.78	2.97	2.86	3.25	3.40	3.14	3.28	3.78	4.01	3.89
After 22 Days:													
$\frac{\Delta V}{V_0} \times 10^2$	Gel fell apart	57.7	43.5	42.9	44.7	54.6	61.3	77.0	94.6	89.4	48.9	28.9	12.5
$C \times 10^2$		6.18	6.83	6.85	6.77	6.33	6.50	5.49	4.95	5.11	6.57	7.64	8.82
$(n_2 - n_1) \times 10^5$		4.49	6.54	7.85	8.85	9.82	11.85	14.25	14.70	14.25	11.80	8.85	4.27
$\frac{n_2 - n_1}{\frac{\Delta V}{V_0} C} \times 10^3$		1.26	2.20	2.67	2.94	2.84	2.97	3.38	3.09	3.11	3.88	4.00	3.90

concentrations of HCl. The swelling produced by the acid is depressed considerably by the addition of NaCl and hence it is possible

TABLE X

Swelling and Double Refraction of 10 Per Cent Gels in Various Concentrations of NaOH Made Up in $\frac{M}{16}$ NaCl

Concentration of NaOH	$\frac{M}{128}$	$\frac{M}{256}$	$\frac{M}{512}$	$\frac{M}{1024}$	$\frac{M}{2048}$
Final pH of outside solution	11.0	10.6	10.0	9.0	6.8
After 7 Days:					
$\frac{\Delta V}{V_0} \times 10^2$	53.9	48.4	43.2	35.3	22.7
$C \times 10^2$	6.34	6.60	6.83	7.26	8.04
$(n_2 - n_1) \times 10^5$	10.94	10.70	10.40	9.35	6.28
$\frac{n_2 - n_1}{\frac{\Delta V}{V_0} C} \times 10^3$	3.21	3.35	3.52	3.65	3.44
After 15 Days:					
$\frac{\Delta V}{V_0} \times 10^2$	59.1	44.2	40.8	34.5	24.3
$C \times 10^2$	6.14	6.80	6.94	7.30	7.94
$(n_2 - n_1) \times 10^5$	11.10	9.78	9.40	8.92	7.16
$\frac{n_2 - n_1}{\frac{\Delta V}{V_0} C} \times 10^3$	3.06	3.25	3.32	3.53	3.71
After 25 Days:					
$\frac{\Delta V}{V_0} \times 10^2$	61.9	42.4	38.5	33.7	24.3
$C \times 10^2$	6.02	6.88	7.06	7.35	7.94
$(n_2 - n_1) \times 10^5$	10.75	10.20	9.05	8.32	7.04
$\frac{n_2 - n_1}{\frac{\Delta V}{V_0} C} \times 10^3$	2.88	3.49	3.33	3.36	3.65

to make the observations over a wide range of pH. As in the case of pure acid the optical modulus of elasticity is reduced by the addition of acid but the effect of acid appears to become of significance only at a pH of the acid below 2.0. At higher pH values the double refraction per unit increase in volume and per unit concentration is practically the same as in the absence of acid. The same is also shown in Table IX where are given data on the swelling and double refraction of 10 per cent gels in various concentrations of H_2SO_4. This acid also lowers the elasticity of gelatin, though it is not as effective as HCl. The effect of the acid on the elasticity of the gels practically disappears at a pH above 2.0.

Alkali affects the elasticity of gelatin in the same way as acids do, but the effect does not appear until the pH of the outside medium is not less than 10.0. This is shown in Table X. The slight drop with time in the swelling of the gels in $\frac{M}{256}$ NaOH and lower is due to the gradual lowering in the pH of the solution which is unavoidable in this range of pH in non-buffered solutions.

The results here on the effect of pH on the elasticity of the gel agree with those found by Sheppard and his coworkers and also by Scarth.[1]

SUMMARY AND CONCLUSIONS

Quincke's researches (1904) have demonstrated that when a 20 per cent gelatin gel is allowed to swell in water it gives rise to positive double refraction, as if the gel were under tensile stresses. If, on the other hand, the gel shrinks on being placed in alcohol it becomes negatively double refractive, as if it were compressed. But the double refraction as found by Quincke lasts only during the process of swelling or shrinking, and disappears as soon as the gel reaches a state of equilibrium.

This phenomenon was investigated here and it was found that the reason for the disappearance of the double refraction is due to the fact that at equilibrium the percentage change in the size of a gel is equal in all three dimensions and the strain is therefore uniform. Double refraction persists as long as there is a difference in the elastic strain in the three dimensions of the strained material.

It was found that when gels are cast on glass slides or in glass frames, so as to prevent swelling in certain directions, the double refraction produced by swelling at 6°C. persists permanently in the gel as long as it is swollen, and is proportional to the percentage change in the linear dimensions of the gel.

Gels made up of various concentrations of isoelectric gelatin of less than 10 per cent when placed in dilute buffer of the same pH as that of the isoelectric point of the gelatin shrink and give rise to negative double refraction, while gels of concentrations of more than 10 per cent swell and give rise to positive double refraction. The double refraction produced in either case when divided by the percentage change in the dimensions of the gel and by its changed concentration gives a constant value both for swelling and shrinking. This constant which stands for the double refraction produced in a gel of unit concentration per unit strain is termed here the optical modulus of elasticity since it is proportional to the internal elastic stress in the swollen gelatin.

It was found that the optical modulus of elasticity is the same both for gels cast on slides and in frames, although the mode of swelling is different in the two forms of gels.

Gels removed from their glass supports after apparent swelling equilibrium, when placed in dilute buffer, begin to swell gradually in all three dimensions and the double refraction decreases slowly, though it persists for a long time. But the double refraction per unit change in dimension and per unit concentration still remains the same as before, thus proving that the internal elastic stress as indicated by the double refraction is brought about by the resistance of the gel itself to deformation.

A study was also made on the effect of salts, acid and base on the double refraction of a 10 per cent gel during swelling. The experiments show that below $\frac{M}{8}$ salts affect very slightly the optical modulus of elasticity of the gel. At higher concentrations of salts the elasticity of the gel is reduced by some salts and increased by others, while such salts as sodium acetate and sodium and ammonium sulfates do not change the elasticity of the gels at all during swelling. The investigated salts may thus be arranged in this respect in the following approximate series: $CaCl_2$, NaI, NaSCN, NaBr, $AlCl_3$, NaCl, Na acetate, Na_2SO_4, $(NH_4)_2SO_4$, Al_2SO_4 and $MgSO_4$. The first five in the series decrease the elasticity while the last two in the series increase the elasticity of the gels during swelling. Acids and bases in higher concentrations exert a powerful influence on the reduction of the elasticity of the gel but in the range of pH between 2.0 and 10.0 the elasticity remains unaffected.

The general conclusions to be drawn from these studies are as follows:

1. Swelling or shrinking produces elastic stresses in gels of gelatin, tensile in the first case and compressive in the second case, both being proportional to the percentage change in the dimensions of the gel.

2. Unsupported gels when immersed in aqueous solutions swell or shrink in such a manner that at equilibrium the percentage change in size is equal in all three dimensions, and the stresses become equalized throughout the gel.

3. Gels cast on glass slides or in frames when immersed in aqueous solutions swell or shrink mostly in one direction, and give rise to unidirectional stresses that can be determined accurately by measuring the double refraction produced.

4. The modulus of elasticity of swelling gelatin gels, as calculated from the double refraction measurements, is the same both for compression and for tension and is proportional to the concentration of gelatin in the gel.

5. The modulus of elasticity of gels during swelling is affected only slightly or not at all by salts at concentrations of less than $\frac{M}{8}$ and is independent of the pH in the range approximately between 2.0 and 10.0.

6. Higher concentrations of salts affect the modulus of elasticity of gelatin gels and the salts in their effectiveness may be arranged in a series similar to the known Hoffmeister series.

7. Acid and alkali have a strong reducing influence on the elastic modulus of swelling gels.

8. The swelling produced in isoelectric gelatin by salts is due primarily to a change brought about by the salts in the osmotic forces in the gel, but in high concentrations of some salts the swelling is increased by the influence of the salt on the elasticity of the gel. This agrees completely with the theory of swelling of isoelectric gelatin as developed by Northrop and the writer in former publications.

9. The studies of Loeb and the writer on the effect of salts on swelling of gelatin in acid and alkali have been in the range of concentrations of salts where the modulus of elasticity of the gelatin is practically constant, and the specific effect of the various salts has been negligible as compared with the effect of the valency of the ions.

In concentrations of salts below $\frac{M}{4}$ or $\frac{M}{8}$ the Hoffmeister series plays no rôle.

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SOLUBILITY CURVES OF MIXTURES AND SOLID SOLUTIONS

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Theoretical Solubility Curves of Mixtures or Solid Solutions of Two Components

The isolation and characterization of biological compounds are attended with special difficulty since some substances, such as enzymes and vitamins, are active in extremely small amount while others, such as the proteins, differ so slightly in either chemical or physical characteristics as to render proof of their purity a difficult problem. It is frequently impossible to decide from analytical results whether the material is a mixture or a solid solution or a pure substance. The phase rule offers the most powerful tool for the solution of such problems. The use of the melting point and boiling point as a test of purity is based on the theory of the phase rule and has been of the greatest help in organic chemistry, but in the case of many substances such as the proteins, these properties cannot be used and it is necessary to make use of an entirely analogous property, the solubility. Thus Landsteiner and Heidelberger (1) were able to show that hemoglobins of closely related animals could be distinguished by solubility measurements, and Sørensen (2) has found that proteins which had been considered as pure compounds for years were, in reality, mixtures or solid solutions of several proteins.

There is an extensive literature (3) on the theory of the solubility of both mixtures and solid solutions but practically all the work has been in relation to the study of mixtures or solid solutions of known components. The problem has been to define the property of the mixture or solid solutions from the properties of the components. In the case of an unknown material, however, the problem is to define the *properties of the components from the solubility of the mixture* and to determine to what extent differences in the solubility, or in the relative amounts of the components, will affect the total solubility or the ratio of the components in the various phases. Without this information it is not possible to say definitely what possible systems may be represented by a given series of experimental results and what may not.

The following discussion is restricted to two components.

The behavior of a system consisting of two solid and one liquid phases may be completely predicted by means of the ordinary phase rule diagram. The phase rule, however, furnishes very little information as to the behavior of a solid solution in contact with a liquid. In this case there are three components and only two phases (assuming no vapor present), so that the system has three degrees of freedom even when the solid phase is present. In order to predict the solubility of a solid solution therefore, or to interpret the curves obtained, it is necessary to assume some relation between the composition of the solid phase and the solubility. The problem, as Van't Hoff and Nernst pointed out (4), is analogous to that of the vapor pressure of a mixture of two miscible liquids, the osmotic pressure in the case of the solution being analogous to the vapor pressure in the case of the liquids. Raoult's law, which states that the vapor pressure of one component of a mixture of liquids is proportional to the mole fraction of that component in the liquid mixture times the vapor pressure of the pure liquid, is the most general theoretical relation between the vapor pressure and composition of liquid solutions. The law holds accurately only in rare cases in which the liquids are closely related, but is a valuable guide in most cases. It will be assumed in the following discussion that the systems obey the phase rule and also Raoult's law. The evidence in favor of the validity of these relations as applied to solid solutions, and especially to proteins, may be summarized.

The Phase Rule.—The question has frequently been raised as to whether the phase rule applied to colloidal solutions in general and to proteins in particular. If the system is in equilibrium the phase rule must be applicable, but it is quite possible that other variables such as the surface energy or diameter of the particles must be considered as well as the usual temperature, pressure, and concentration. If this were true the rule would be of little practical value. The work of Sørensen (2) and of Cohn (5) has shown, as a matter of fact, that proteins behave just as other compounds and that the solubility curves are those predicted for such systems by the phase rule. The results obtained with pepsin (6) also agree with the phase rule. Since the phase rule applies only to systems at equilibrium it is necessary to be sure that the values obtained are equilibrium values. In the case of solubility relations this can best be done by approaching equilibrium from the supersaturated and also from the undersaturated side. If these two values agree they must represent an equilibrium value. A second point of great importance is the choice of components. In the case of proteins dissolved in salt solution, for instance, the entire salt solution, no matter what its composition, may be considered as one component provided the composition of the salt solution is always the same, *i.e.*, the precipitate contains no salt nor water. Under these conditions a solid protein in equilibrium with a salt solution consists of two phases and two components and has therefore two degrees of freedom, so that if the temperature and pressure are fixed the concentration of the solution is fixed and cannot be varied by varying the quantity of either phase. If, however, the solid phase contains water or some of the ions or salts present in solution, as well as the protein, then the system has three components since three values, water, salt, and protein, are needed to express the composition of the phases; and the system has three degrees of freedom even in the presence of the solid phase. That is, the concentration will vary with the amount of solid present as well as with the temperature and pressure. Sørensen has found that the neglect of this possibility was the cause of the apparently abnormal results obtained by earlier workers in connection with the solubility of egg albumin.

Solid Solutions and Raoult's Law.—Raoult's law predicts that the solubility (vapor pressure) of a mixture of two miscible liquids (or solids) will be represented by a line connecting the vapor pressure (solubility) of the two pure components. The study of isomorphous mixtures of isomers has shown that the solubility of such mixtures may sometimes be predicted from Raoult's law, as in the case of the camphor oximes (7); but that in other cases the solubility of such mixtures may show a maximum or minimum, which is contrary to

Raoult's law. Landsteiner and Heidelberger (1) have found that mixtures of hemoglobins from two closely related animals have solubilities between those of the two separate proteins, so that they form solid solutions and agree qualitatively at least with Raoult's law. Sørensen's (2) results also indicate that solid solutions are of common occurrence with proteins, so that it is a possibility which must always be considered. There is, however, no reason to suppose that Raoult's law holds in general in case of solid solutions and predictions made from it must be considered only as approximations.

Solubility Curves of Mixtures from the Phase Rule

The solubility of mixtures of various proportions of two substances in a liquid may be represented conveniently on the ordinary phase rule diagram when the composition of the mixtures is known. In the case of an unknown mixture the problem is to derive the solubilities and relative amounts of the components of the mixture from the solubility curve of the mixture when added in increasing amount to the liquid. This can be done if it be further assumed that the mixtures form ideal solutions, *i.e.*, that the solubility of one is independent of the presence of the other. Assume that a solid material consists of a mixture of A moles of A and FA moles of B. The ratio of B to A is then F. The solubility of A is a and of B is Ca, A is the less soluble so C is greater than 1. Increasing amounts of the mixture are added to unit volume of liquid and the total amount of solid in the solution determined.

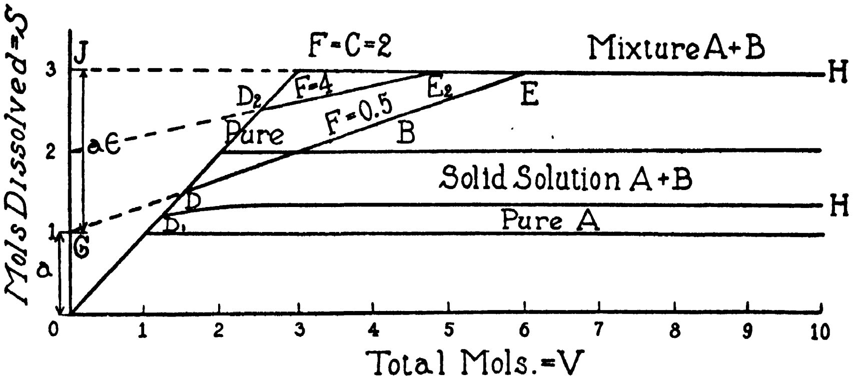

FIG. 1

This amount is plotted as ordinate against the total amount of the mixture added as abscissa (Fig. 1). At first all the solid added will dissolve and the points will fall on a line with a slope of 45° (0-D). As soon as an amount of A or B equal to its solubility is added, solid will appear and there will be a break in the curve as at D. The system has now two phases and three components and the concentration can still change with the amount of the phases present. As more solid is added the solubility will continue along the line D-E, until at E the second solid phase appears. There are now three phases and three components, so if the temperature and pressure are fixed the composition is fixed and there will be no further change in concentration with added solid. It is now possible to determine F, C and a, either graphically or algebraically.* D-E and E-H are continued back to the solubility axis. The distance $0G$ is therefore a or Ca and GJ is either Ca or a. Since C, by definition, is more than one the larger of these values is Ca and the smaller is a. The slope of the line D-E is $\frac{F}{1+F}$ if $0G = a$ and $\frac{1}{1+F}$ if $0G = Ca$. When $F = C$ both solids appear together so that the solubility curve shown in Fig. 1 for $F = C$ has the same form as for a pure substance. If $F > C$, B (the more soluble) will appear first as solid while if $F < C$, A (the less soluble) will appear first. The diagram shows also that in the part of the curve between D and E the solid phase will be either pure A or pure B and that the extent of the range over which the pure solid is present depends on the difference between C and F and is zero when $C = F$. Evidently then this is the most significant part of the curve for deciding whether or not a given material is a mixture and analysis of the first precipitate to appear is the most sensitive test. If this agrees with that of the original material, or if its solubility is the same as that of the original material, then either there is very little of a second substance present or the solubilities of the two are very nearly in proportion to their relative amounts. In case it were suspected that one property of a material were due to one substance and another property to another substance the ratio of the two properties in the first precipitate to appear would serve as a very sensitive test, since at that point the ratio of A to B must differ greatly from that of the original mixture even though the amount of B

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* See equations on pages 281 and 282.

is so small as not to affect the shape of the total solubility curve and *the range of existence of pure A or B so small as to render it impossible* to actually isolate pure A or B. Still, near this range the ratio of A to B would be different from that in the original mixture. If the ratio of A to B may be measured therefore the possibility of a small per cent mixture may be ruled out and the material must be either a pure substance or the ratio of the solubilities of the two components must be very nearly identical with their relative amounts. If in addition, this were found to be the case in several solvents it would be practically certain that the material was a pure substance since it is highly improbable that the relative solubilities of two substances (except allotropes or polymorphs) should be identical in various solvents.

Solid Solutions

The question as to whether a given material is a mixture may therefore be settled with practical certainty by solubility measurements in various solvents. Unfortunately, however, in the case of solid solutions the conclusions are much less definite. All that can be learned from the phase rule is the point at which the solid will appear if the relation connecting the solubility of the solid solution in terms of the solubility of the pure components and of the percentage composition is known. Since in this case there is only one solid phase the system will still have three degrees of freedom after the solid appears and the concentration in the solution will vary with the amount of solid phase. No information as to the nature or extent of this variation can be gained from the phase rule alone. If, however, the system be assumed to follow Raoult's law and the relative proportions and solubilities of the two components are known, then the form of the solubility curve and the ratio of A to B at any point can be calculated. Conversely, if the solubility curve is known the relative solubilities can be calculated. In order to define the system, however, it is necessary to know three points on the solubility curve in the case of a solid solution instead of two as in the case of a mixture.

Form of the Solubility Curve.—The curve for the total solubility resembles that for the case of a mixture but has only one sharp break at D. From D on the curve rises, the slope becomes less as more and more mixture is present, and approaches zero asymptotically. The curve will lie between that for pure A and pure B and will approach the curve for either one or the other as F varies from zero to infinity. If $C = 1$ the curve will have the form of that for a pure substance and the total solubility will be independent of the amount of the solid present. In this case the ratio of A/B would be the same at all points and there would be no indication of the presence of more than one substance. If C has any value but one, however, the ratio of A/B in the first precipitate that appears will be $1/FC$ while that of the original solid solution is $1/F$. As the amount of solid present increases the ratio of A/B in the solid approaches $1/F$ while the ratio of A/B in solution approaches $1/FC$. At all points the ratio of A/B in solution to A/B in the solid equals $\frac{1}{C}$. As in the case of the mixture therefore the best test for the presence of two hypothetical components is the ratio of the two properties in the solid, when the material is nearly all dissolved, as compared with the ratio in the solution when a large amount of solid is present.

These conclusions may be obtained algebraically from Raoult's law as outlined below.

Mixtures

A the less soluble

$$A = \text{moles } A = \frac{V}{1 + F}$$

$$FA = \text{moles } B = \frac{VF}{1 + F}$$

a = solubility A

Ca = " B

$(C > 1)$

Total moles dissolved $= S$

Total moles in system $= A + FA = V$

Total volume $= 1$

Then

From $0 - D$

$$V = S \tag{1}$$

From $D - E$

$$S = a + \frac{VF}{1 + F}, \text{ if (Intercept) } 0\,G < G\,J \tag{2}$$

or

$$S = Ca + \frac{V}{1 + F}, \text{ if (Intercept) } 0\,G > G\,J \tag{3}$$

From $E - H$, $S = a + Ca$

or at D, $S_D = V_D$

$$F = \frac{S_D}{a} - 1$$

or

$$F = \frac{Ca}{S_D - Ca}$$

From (2) or (3)

When $V = 0$

$$S = a \text{ or } Ca = 0\,G \tag{4}$$

Conditions determining which solid appears first.

V_A = value of V when A appears

V_B = " " " " B "

Then $\dfrac{V_A}{1 + F} = a \qquad \dfrac{V_B\,F}{1 + F} = Ca$

$$V_A = a\,(1 + F) \tag{5}$$

$$V_B = \frac{Ca}{F}\,(1 + F) \tag{6}$$

Divide (6) by (5)

$$\frac{V_B}{V_A} = \frac{C}{F}$$

$\therefore$ if $F < C$, $V_B > V_A$ and A appears first

" $F = C$, $V_B = V_A$ " both appear together

" $F > C$, $V_B < V_A$ " B appears first

Solid Solutions

x = moles A in solution

y = " B " "

$x + y = S$

Raoult's law

$$x = \frac{\left(\frac{V}{1 + F} - x\right) a}{\left(\frac{V}{1 + F} - x\right) + \left(\frac{VF}{1 + F} - y\right)} \tag{1}$$

$$x = \frac{\frac{a\,V}{1 + F} - a\,x}{V - S} \tag{1 a}$$

$$y = \frac{\left(\frac{VF}{1 + F} - y\right) Ca}{\left(\frac{VF}{1 + F} - x\right) + \left(\frac{VF}{1 + F} - y\right)} \tag{2}$$

$$y = \frac{\frac{Ca\,VF}{1 + F} - Ca\,y}{V - S} \tag{2 a}$$

$$x = \frac{a\,V}{(1 + F)} \cdot \frac{1}{(V - S + a)} \tag{3}$$

$$y = \frac{a\,V}{1 + F} \cdot \frac{C\,F}{V - S + Ca} \tag{4}$$

$$S = x + y = \frac{aV}{1+F}\left(\frac{CF}{(V - S + Ca)} + \frac{1}{(V - S + a)}\right) \tag{5}$$

$$\frac{x}{y} = \frac{1}{FC} \cdot \frac{V - S + Ca}{V - S + a} \tag{6}$$

From (5) a, F and C may be solved numerically from 3 points on the solubility curve.

When $V - S \doteq 0$ (very little solid)

$$S_D = \frac{aC(1 + F)^*}{C + F}$$

and when large excess solid ($V >> S$)

$$S_H = \frac{a(1 + FC)}{1 + F}$$

also, if $C = 1$

$$S = a$$

From (6) $V - S \doteq 0$

$$\frac{x_D}{y_D} \doteq \frac{1}{F}$$

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* This relation is derived as follows:

Let $V - S = U$; also let S_D be the value of S when U approaches zero; *i.e.*, $S_D \doteq V_D$

From (5) $U = S_D\left[1 - \frac{a}{1+F}\left(\frac{CF}{U + Ca} + \frac{1}{U + a}\right)\right]$

$$\text{or } S_D = \frac{U}{1 - \frac{a}{1+F}\left(\frac{CF}{U + Ca} + \frac{1}{U + a}\right)} \doteq \frac{0}{0}$$

The value of S_D is found by differentiating the indeterminate fraction with respect to U and finding its limiting value as U approaches zero.

and if $V - S \doteq V$ (large excess solid)

$$\frac{x_H}{y_H} \doteq \frac{1}{F\,C}$$

also if $w = A$ in solid $= \dfrac{V}{1 + F} - x$

$$z = B \text{ in solid } = \frac{VF}{1 + F} - y$$

Substitute in (1) and (2)

$$x = \frac{w\,a}{w + z}$$

$$y = \frac{Ca\,z}{w + z}$$

$$\frac{\dfrac{x}{y}}{\dfrac{w}{z}} = \frac{1}{C}$$

SUMMARY

It is shown that the relative amounts and the solubilities of the two components of a mixture or solid solution may be calculated from the solubility curve of the mixture or the solid solution.

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CRYSTALLINE PEPSIN

I. ISOLATION AND TESTS OF PURITY

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I

INTRODUCTION

Enzymes are in many respects connecting links between living and inanimate matter since their action is analogous to inorganic catalysts, although the enzymes themselves are found only in living organisms. As catalysts they increase the rate of one or more specific reactions and so act as directive agents for the reactions occurring in the organism. This directive property is undoubtedly essential for the existence of living cells. As a consequence of these properties the study of enzymes has been of interest to both chemists and biologists and has resulted in a great increase in the knowledge of their mode of action. The results of attempts to isolate the enzymes in pure form, however, have been singularly unsuccessful. There seems to be no convincing evidence that any enzyme has been obtained in the pure state; and only one, the urease described by Sumner (1), has been previously obtained in crystalline form. A number of methods have been found which allow the activity of an enzyme preparation to be increased almost indefinitely; at the same time, however, the preparation becomes more unstable and eventually the activity becomes lost.

In practically all the work the assumption has been made either explicitly or otherwise that the activity was a measure of the purity of the preparation and that any increase in activity was due to an increase in purity. This is not necessarily true. If the enzymes are analogous to inorganic catalysts then it is quite possible that the activity depends on the physical arrangement of the molecules or atoms (2). Evidence for this relation between the physical state and the activity was found by Fodor (3) in the case of the proteolytic enzymes of yeast, and by Kuhn and Wasserman (4) in the case of hemin. It is possible, on the other hand, that enzymes in general are of the type of hemoglobin (which might be considered an enzyme), and that they consist of an active group combined with an inert group. It might be possible under certain conditions to attach many more active groups to the inert group and so increase the activity above that of the original compound. Either of the above ideas would account for the well-known fact that crude preparations are much more stable than purified material and that the rate of inactivation of enzyme solutions practically always shows evidence of a mixture of stable and unstable forms (5, 6).

There is some reason to think, therefore, that enzymes exist in a more stable form for either physical or chemical reasons, and in view of the uniformly negative results which have been obtained in attempting to isolate the most active preparations it seemed advisable in attempting the isolation of pepsin to study the more stable as well as the most active fraction.

II

PRELIMINARY EXPERIMENTS

A number of methods have been proposed for the purification of pepsin, such as precipitation with safranin (7), etc., fractionation by various adsorbents, and precipitation by dialysis from acid solution (Pekelharing (8)). These and a number of other methods were tried and more or less active preparations obtained. The results with Pekelharing's method seemed the most encouraging, however, since the loss of activity was less and there was some indication that a constant activity was reached. This result has been reported by Pekelharing and also by Fenger, Andrew and Ralston (9) using a similar method. It was found, however, that the dialysis could be dispensed with and the process made more rapid and efficient by solution with alkali and subsequent precipitation with acid, after a preliminary precipitation with half saturated $MgSO_4$ or $(NH_4)_2SO_4$. The amorphous material so obtained contains about half the activity present in the original material and is 3 to 6 times as active as measured by the liquefaction of gelatin and about 5 times as active as measured by the digestion of casein or by the rennet action on milk.[1] Repetition of this procedure gave products of increasing activity as measured by the liquefaction of gelatin, and apparently this activity could be increased indefinitely. Several samples were obtained which were 100 times as active as the original preparation. They were also more unstable, so that each succeeding precipitation was accompanied by a larger and larger percentage loss until finally no more active material remained. This has been the fate of all previous attempts to isolate the most active fraction of a number of enzymes. When the activity of the various fractions was determined by the rate of hydrolysis of casein or by the rennet action on milk, however, it was found that the activity increased until it reached about 5 times that of the crude preparation and then remained constant instead of increasing as did the gelatin liquefying power. This was the result reported by Pekelharing and also by Fenger, Andrew and Ralston. This material appeared to be protein, as previous workers had found, and was reasonably stable. Efforts were therefore made to isolate this protein in crystalline form.

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[1] *Determination of Activity.*—The rate of digestion of proteins by pepsin may be followed by determining the increase in carboxyl groups or amino nitrogen, or by the decrease in protein nitrogen or by the changes in viscosity. The increase in carboxyl groups probably represents the best measure of the progress of the reaction and has been used as the basis of the activity units used in this paper.

The most widely used unit of enzyme activity in general is that suggested by Euler (16) and is equal to the velocity constant of the reaction divided by the grams of enzyme. Theoretically this is undoubtedly correct since the activity of a catalyst can be expressed only as a velocity constant. Many enzymes do not give a velocity constant independent of the substrate concentration but instead the velocity constant decreases as the substrate concentration increases. This is especially true of pepsin and in addition the reaction does not follow any simple reaction rate. The end point of the reaction, which must be known in order to calculate the velocity constant, is also very difficult to determine. It is not practical therefore to use the velocity constant as a measure of activity of pepsin.

The activity of pepsin may conveniently be defined as the milliequivalents of carboxyl groups liberated per mole or gram of enzyme per minute at 35.5°C., optimum pH, and 5 per cent substrate concentration. Theoretically it would be better to use that substrate concentration at which the rate of reaction is a maximum, but experimentally this is difficult, owing to the high viscosity of such concentrated solutions. The enzyme concentration used should be in the range in which the activity is proportional to the enzyme concentration. In order to determine the activity in this way the rate of hydrolysis of casein, gelatin, edestin, and denatured egg albumin at pH 2.0 to 2.5, was determined by means of the increase in formol titration (19).

The following abbreviations are used in this paper:

PU = proteolytic units = milliequivalents carboxyl groups per minute.
$[\mathrm{PU}]_{\mathrm{gm.}}$ = " " per gram.
$[\mathrm{PU}]_{\mathrm{gm.}}^{\mathrm{cas.F}}$ = " " " " as determined by cas. F. method, etc.

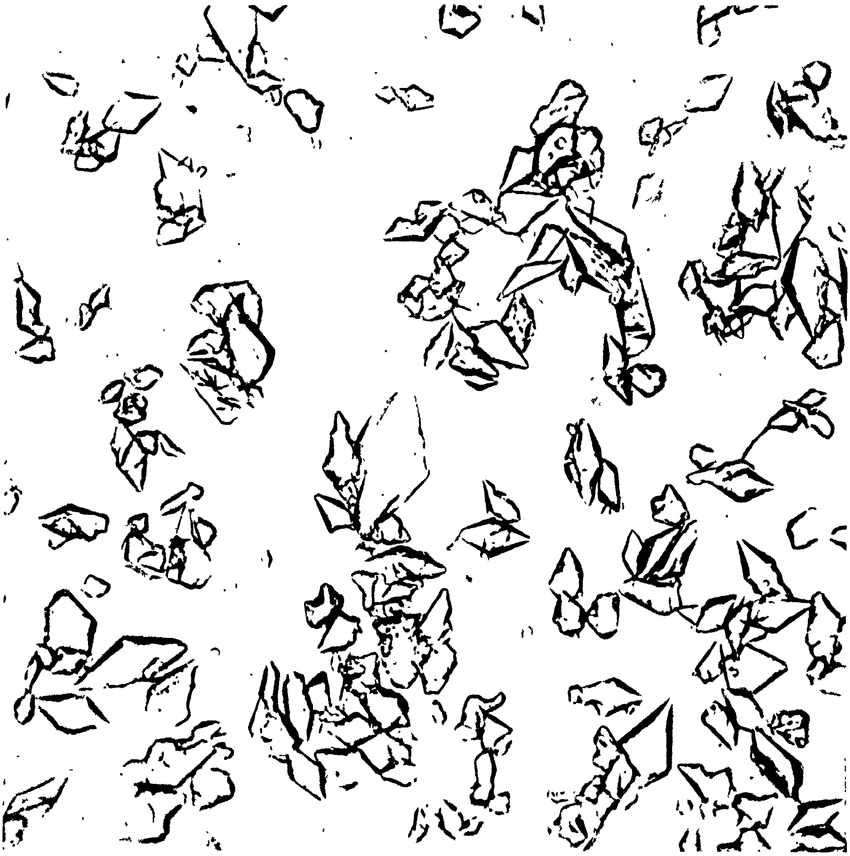

FIG. 1. Crystalline pepsin

III

Isolation of the Crystalline Enzyme

It was noticed that the precipitate which formed in the dialyzing sac when the procedure of Pekelharing was followed appeared in more or less granular form and filtered rather easily, as though it were on the verge of crystallization. This precipitate dissolved on warming the suspension and it was eventually found that it could be induced to crystallize by warming to 45°C., filtering, and allowing the filtrate to cool slowly. The crystals so obtained were regular hexahedra and showed a tendency to grow in clusters, especially when appearing from more acid solutions. They are remarkably similar to the urease crystals pictured by Sumner and differ only in that they have a hexagonal base while the urease has an octagonal base. On one occasion a few crystals with truncated pyramids were obtained. They had the same activity and optical activity as the usual form. The crystals showed positive double refraction and were optically active in solution. They possessed proteolytic activity, when dissolved, equivalent to 5 times that of the U. S. P. 1 to 10,000 pepsin as measured by hydrolysis of casein, and 2.5 times as measured by the liquefaction of gelatin.

Improved Method for the Preparation of the Crystals.—The isolation of the crystals in bulk by the above method was difficult owing to the dialysis. It was found that this could be avoided and the purification carried out as outlined above for the amorphous preparations except that the acid precipitate was dissolved at 45° C. in concentrated solution. On inoculation, this solution set to a solid paste of crystals. The following is an outline of the method as finally developed.

IV

General Properties and Analysis of the Crystals

The material prepared in this way has the general properties of a protein. It is coagulated by heat, precipitated by saturation of the solution with $MgSO_4$ or $(NH_4)_2SO_4$ and gives a strongly positive xanthoproteic test. The Millon test is negative. The crude material contains a large amount of yellowish pigment which is removed with difficulty. It may be largely removed by reprecipitation with $MgSO_4$ and becomes less as the material is recrystallized. All the preparations however, give a slightly yellowish solution when dissolved although the dry crystals are pure white after several crystallizations. There is no relation between the activity and the color. Analysis of the material gave the results shown in Table II.

The crystals are difficult to dissolve after drying and are best kept under saturated $MgSO_4$ at 5°C. They are instantly inactivated by alkali in solution and lose activity slowly in acid solutions. The

TABLE I

Preparation of Crystalline Pepsin

Procedure	Activity per gm. dry weight Method			
	Gel. V.	Cas. S.	Rennet	E. A.
500 gm. Parke, Davis pepsin U. S. P. 1:10,000 dissolved in 500 ml. H_2O and 500 ml. 1 Normal H_2SO_4 added. 1,000 ml. saturated $MgSO_4$ added with stirring. Solution filtered through fluted paper (S. and S. No. 1450½) and then with suction. Filtrate discarded.	0.075	2.5	2.5	4.5
Precipitate 1. Wash twice with equal volume ⅔ saturated $MgSO_4$, filter with suction. Filtrate discarded.				
Precipitate 2. Stir with water to thick paste and M/2 NaOH run in until complete solution. (Great care must be taken to avoid local excess of NaOH. pH never more than 5.0.) M/2 H_2SO_4 added with stirring until heavy precipitate forms, (pH about 3.0), 3 to 6 hrs. at 8°C., filter with suction. Filtrate discarded.	.15	7.5	7.5	7.0
Precipitate 3. Stir with H_2O to thick paste at 45°C., M/2 NaOH added carefully until precipitate dissolves, (filter if cloudy and discard precipitate). Beaker containing filtrate placed in a vessel containing about 4 liters of H_2O at 45°C., inoculated and allowed to cool slowly, cooling should require 3 to 4 hrs. and heavy crystalline precipitate should form at about 30 to 35°C. Solution kept at 20°C. for 24 hours. Thick crystalline paste, filter with suction.	.15	10.0	10.0	8.0
Precipitate 4. Wash with small amount of cold H_2O and then with ½ saturated $MgSO_4$ and store under saturated $MgSO_4$ at 5°C.	.17	14.0	[14.0]	9.0
Filtrate. M/2 H_2SO_4 added to pH 3.0, amorphous precipitate filtered off and treat as Precipitate 3.				

Recrystallization

Method 1. Crystalline paste filtered with suction on large funnel so as to form a thin layer of crystals and washed 3 times with cold M/500 HCl. Filter cake stirred to a paste with ½ its weight of water, the suspension warmed to 45°C. and M/2 NaOH run in slowly with constant stirring until the precipitate dissolves ($pH < 5.0$). M/2 H_2SO_4 is then run in until the solution is faintly turbid, a few crystals added and the solution allowed to cool slowly as before. A heavy crop of crystals should separate in about 24 hrs. The suspension is then warmed to 45°C. again and more H_2SO_2 added until the pH of the suspension is about 3.0. It is then allowed to cool slowly again, and filtered after 24 hrs. The crystals may be washed with M/500 HCl until free of SO_4.

Ammonium sulfate may be used in place of $MgSO_4$. Sodium acetate may be used in place of sodium hydroxide.

Method 2. Crystals dissolved with NaOH and treated as described for Precipitate 2.

inactivated material is digested by the remaining active material and a large amount of tyrosin crystallizes out. This process also occurs slowly in the ice box so that the crystals on standing become mixed with nonprotein material that is not precipitated by salt nor by heat. The crystals can be freed from this soluble material by thorough washing with H_2O or by recrystallization. When freshly prepared in this way 98 to 99 per cent of the nitrogen is precipitated from solution by heating rapidly to boiling at pH 3 with sulfuric acid and Na_2SO_4, by saturation with $MgSO_4$ or $(NH_4)_2SO_4$, by the addition of alkali and subsequent neutralization, or by heating with 10 per cent trichloracetic acid.

TABLE II

Analysis. Dried at 60° in vacuo for 24 Hrs.

Method......................	Dumas	Kjeldahl	Van Slyke							Precipitation with $BaCl_2$
	Total N	Total N	Amino N	C	H	Cl	S	P	Ash	$SO_4^{=}$
Per cent dry weight	15.5	15.15	0.80	52.3	6.66	0.23	0.88	0.078	0.40	0
	15.3	15.30		52.6	6.64	.20	.82		.55	
					6.70					

TABLE III

Activity and Composition of Various Preparations of Pepsin, Crystallized Once

Preparation......................	1	2	3-5	5-10	10-12	12-20	22
$[PU]^{gel. V.}_{gm.}$..............	0.17	0.16	0.18	0.154	0.16	0.14	0.17
Per cent N...............	15.2	15.1	15.1	15.3	15.2	15.0	15.1
$[\alpha]^{22°}_{D}$ pH 4.5.............	−70	−72	−74	−73	−74	−71	−70

Constant Activity of Various Preparations.—About 2 kg. of the crystals have been prepared from six different lots of the commercial preparation during the course of this work and have all had the same percentage of nitrogen and the same activity within the experimental error. A summary of these properties of the various preparations is given in Table III. The preparations in some cases were combined at the final crystallization.

The relative activity with various substrates and various methods is shown in Table IV. The figures are the average of 6 to 10 determinations with different enzyme preparations. All crystalline preparations tested yielded the same result with these methods, but amorphous preparations frequently gave much higher activity as measured by gelatin hydrolysis and lower activity as measured by egg albumin, while the activity as measured by casein hydrolysis remained constant. On recrystallization of these abnormal amorphous precipitates the activity always returned to the characteristic value for the crystalline material, shown in the table.

TABLE IV

Activity of Various Enzyme Preparations

Enzyme	Substrate	Method	Activity. Milliequivalents per gm. per min. = $[PU]_{gm.}$	Milliequivalents per millimole or equivalents per mole per min. = $[PU]_{mole}$
Crystalline pepsin	Casein	Formol	14 ± 0.5	500
		Solution	[14]	
		Rennet action	[14]	
	Gelatin	Formol	.17 ± 1	6
		Viscosity	[.17]	
	Edestin	Formol	28 ± .8	1,000
	Egg albumin	Formol	9 ± .5	320
U.S.P. pepsin 1:10,000	Casein	Formol	2.5	
		Solution	[2.5]	
		Rennet action	[2.5]	
	Gelatin	Formol	.075	
		Viscosity	[.075]	
	Egg albumin	Formol	4.5	

The activity compared to other enzymes is less than that reported by Sumner for urease, on a weight basis, and much less than for several other enzymes, but comparison should be made on the basis of activity per mole rather than per gram of enzyme.

V

Evidence Concerning the Purity of the Crystalline Material

Constant Activity and Composition on Repeated Crystallization.—The work reported above shows that a crystalline protein having proteolytic activity may be obtained in quantity from commercial preparations of pepsin. The question of interest is whether this proteolytic activity is a property of the protein molecule or of another molecular species associated with the protein. The usual criterion for the purity of a substance is constant composition and properties after repeated recrystallization. Unfortunately in the case of proteins the melting point, which is the most sensitive test of purity, cannot be used since proteins decompose before melting. Of the other properties the proteolytic activity, optical rotation, and percentage of nitrogen and of phosphorus were considered the most significant and these properties were determined on preparations obtained from a series of seven successive crystallizations. A summary of the results of this experiment is shown in Table V. There is no perceptible drift in any of the properties determined and therefore no indication that the material can ever be separated into fractions by crystallization under these conditions. This shows that the composition of the crystals is independent of the concentration and quantity of the solution from which they are formed.

TABLE V

Summary of Recrystallized Pepsin

Crystallization No. and color	Quantity of crystals	$[PU]^{\text{gel. V.}}_{\text{gm.}}$	$[PU]^{\text{cas. S.}}_{\text{gm.}}$	N	P	$[\alpha]^{22°}_{D}$ pH 5.0	Formol per gm.
	gm.			*per cent*	*per cent*		*ml. 0.1 NaOH*
1. Dark brown........	50	0.18	14.0	14.85	0.078	−70	14.0
2. Brown.............	22	.17	14.5	15.0	.080	−70	12.5
3. Yellowish...........	15	.15	15.0	15.14	.075	−68	12.5
4. Slightly yellow fine powder...........	9	.16	14.5	15.16	.076	−67.6	11.0
5. Nearly white........	5	.16	14.8	15.13	.077	−68	11.0
6. " "	3	.14	14.5		.073	−72	11.0
7. " "	.5	.17	15.0	15.15	.080	−72	12.0
Original preparation....		.075	2.5				

Several systems could give this result (10). (1) A mixture of two or more substances present in amounts which are proportional to their solubilities. (2) A solid solution of such composition as to have a minimum solubility. This would correspond to a constant boiling point mixture of miscible liquids. (3) A solid solution of two or more substances having nearly the same solubility. (4) A pure substance.

The various possibilities noted above all depend on some definite relation between the composition of the mixture and the solubilities of the hypothetical components. If this relation were changed, the composition would be changed (except in the case of allotropic modifications). The possibility of such a relation would be greatly reduced if the recrystallization were carried out in a number of different solvents in which the solubility was different. It is quite possible that a mixture of constant composition might be obtained from one solvent just as constant boiling mixtures are frequently found, but it would be expected that the composition of the mixture would vary with different solvents just as the composition of constant boiling mixtures varies with the pressure. If it could be shown therefore that the material retained its constant proteolytic power and composition after recrystallization from a number of different solvents, it would be excellent proof that it was a pure substance. Unfortunately, however, it is not possible to crystallize the material from a series of solvents, but the same result may be obtained by studying the solubility in several solvents.

Results of the Solubility Determinations.—The values for the solubility obtained with different amounts of precipitate in various solvents are shown graphically[2] in Figs. 2 to 6. Since the solubility is found to be independent of the amount of precipitate this procedure does not affect the shape of the curve. The soluble nitrogen per milliliter of solution, the proteolytic activity per milliliter of solution as determined by the digestion of casein or gelatin ($[PU]_{ml.}$) and the optical rotation of the solution in a 1 dm. tube are plotted against the total nitrogen content per milliliter of the suspension. The values corresponding to solutions obtained from the undersaturated side are represented by solid dots and those corresponding to solutions obtained from the supersaturated side are represented by circles. The solid lines drawn for the curves representing the pepsin per milliliter and the optical activity were calculated on the assumption that the ratio of activity or optical activity to nitrogen was the same in the solution as in the original crystals. That

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[2] In order to save space the scale representing the total amount of nitrogen per milliliter of suspension has been changed to a logarithmic one in some of the curves after the amount has become large as compared to the amount in solution.

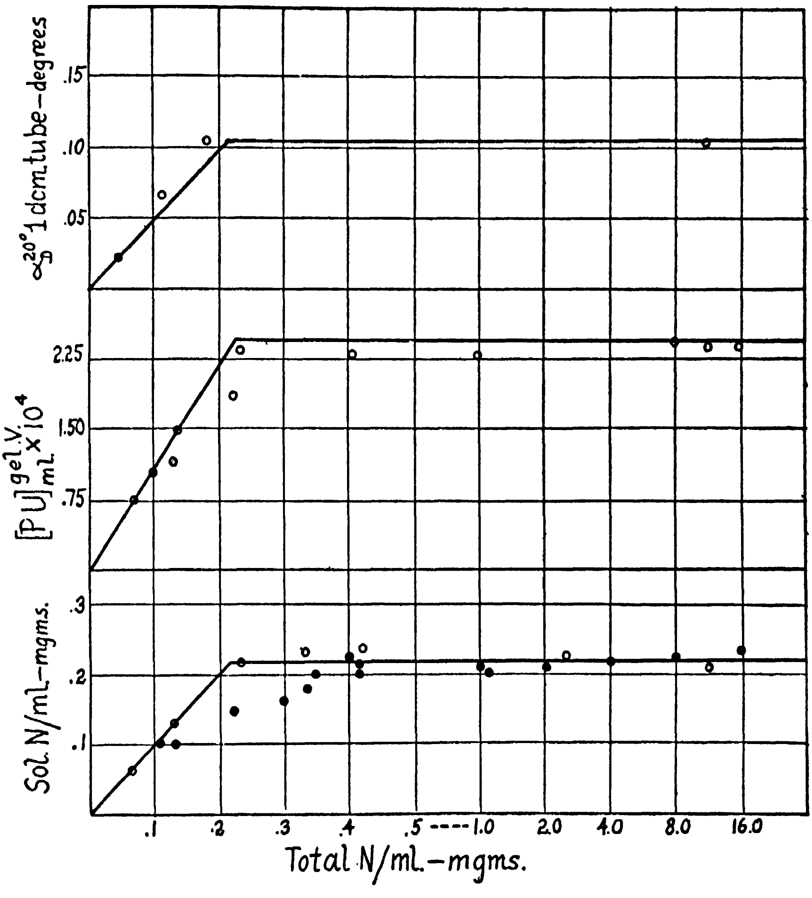

FIG. 2. Solubility of crystalline pepsin in 0.05 acetate and 0.5 saturated magnesium sulfate at 8°C.

is, 1.15 proteolytic units per gram of nitrogen as measured by the liquefaction of gelatin, 92 proteolytic units per gram of nitrogen as measured by the digestion of casein and a specific rotation per gram of nitrogen of −460° as measured at 22 to 24°C. with the sodium D line.

The results show: (1) that the solubility is independent of the quantity of solid present, (2) that the value for the solubility is an equilibrium value since it is the same whether obtained from supersaturated or undersaturated solutions, and (3) the specific proteolytic activity and the specific optical activity are the same for the material present in the solution as for the original material. The material behaves in regard to these determinations as a pure substance and there is no evidence of mixture. They show conclusively that the activity cannot be ascribed to the presence of a minute amount of a very highly active material associated with the protein, unless it be further assumed either that this active material has about the same solubility as the protein or that it forms solid solutions with the protein. If the active material were soluble and *mixed* with the protein the activity per milliliter of solution

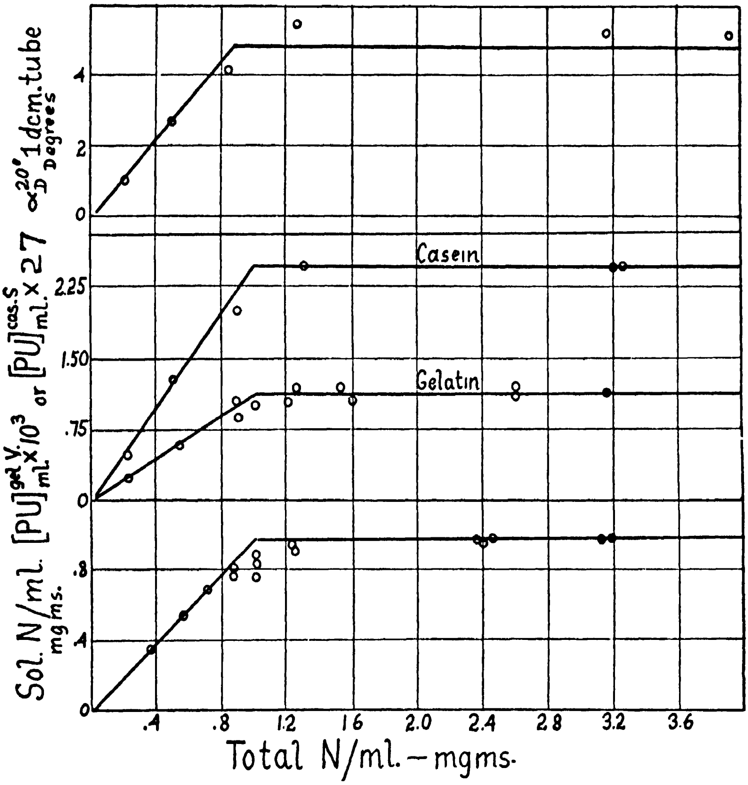

FIG. 3. Solubility of amorphous pepsin in 0.05 acetate and 0.5 saturated magnesium sulfate at 20°C.

would continue to increase as more and more solid material was added. While if it were present as a relatively insoluble material the activity per milliliter of the supernatant solution would become constant before the solubility of the protein had become constant. The figures show, however, that the proteolytic activity of the liquid becomes constant at the same point at which the concentration of the protein in the solution becomes constant. The only mixture which would behave in this way would be one in which the relative amounts of active material and protein were in almost exact proportion to the relative solubilities of the two substances. This might conceivably be the case in one solvent but the possibility that this relation would hold in several solvents appears so small as to be negligible.

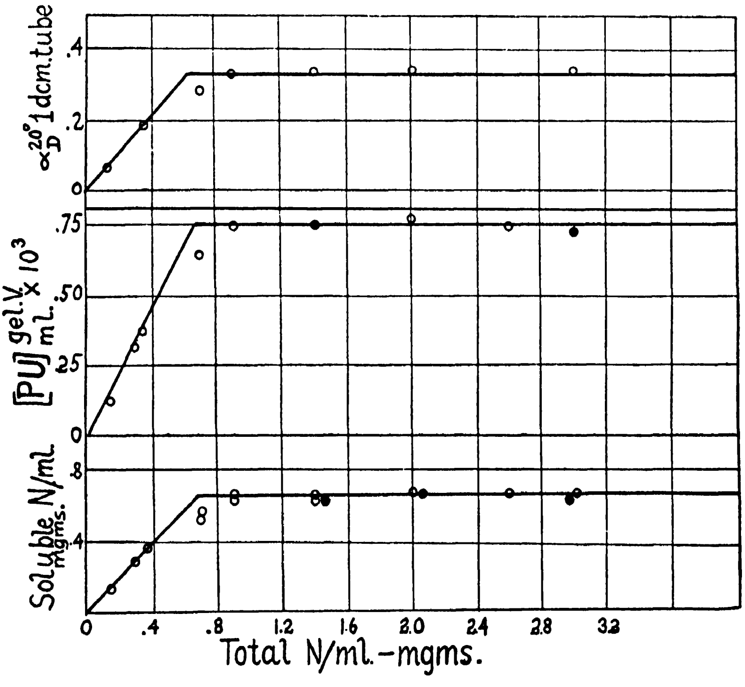

FIG. 4. Solubility of amorphous pepsin in 0.01 acetate and 0.5 saturated magnesium sulfate at 20°C.

There remains only the possibility that the active material forms a solid solution with the protein and is either present in small amount or has about the same solubility as the protein. If it were present in small amount and dissolved in the protein, its presence would not affect the total solubility curve. The ratio of the active material to the protein in the precipitate at the point where the precipitate first

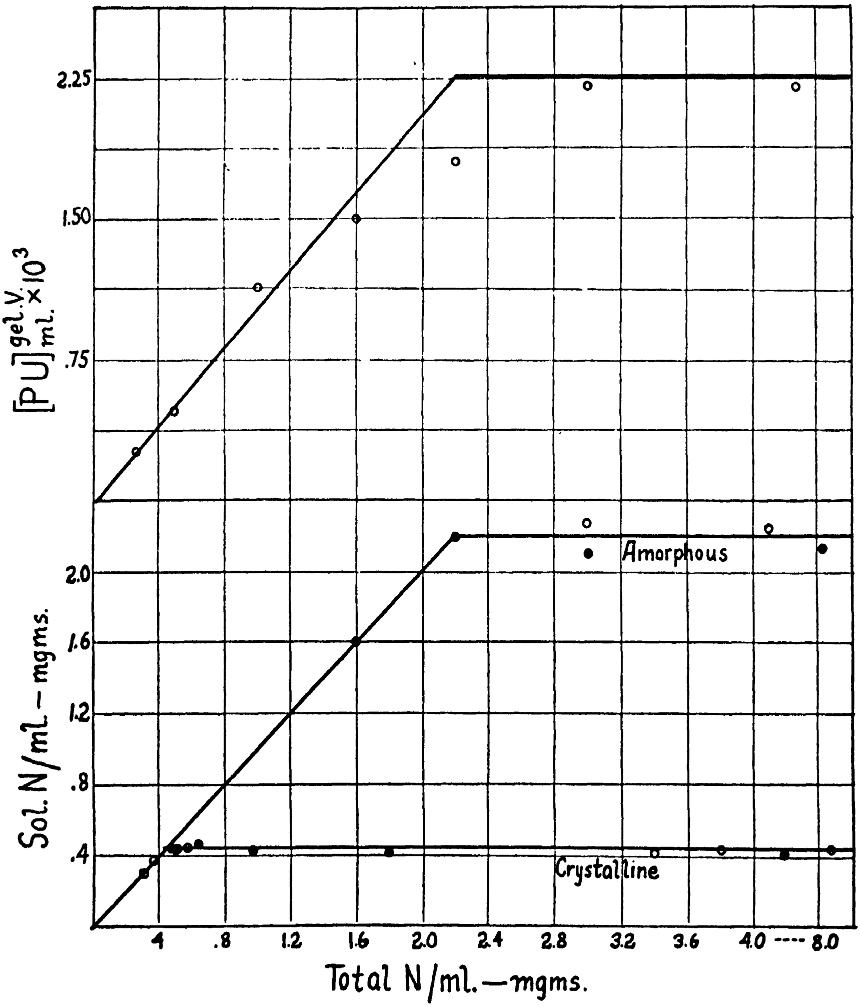

FIG. 5. Solubility of crystalline and amorphous pepsin in 0.055 acetate and 0.444 saturated magnesium sulfate at 20°C.

appears would differ, however, from the ratio in the original material. The ratio in the solution in that part of the curve where there is a large amount of solid present would also differ from the ratio in the original material. The figures show that this latter condition does not occur. The activity per gram of nitrogen in the solution in equilibrium with a large excess of precipitate is the same within the experimental error as the ratio in the original material. The activity-nitrogen ratio of the

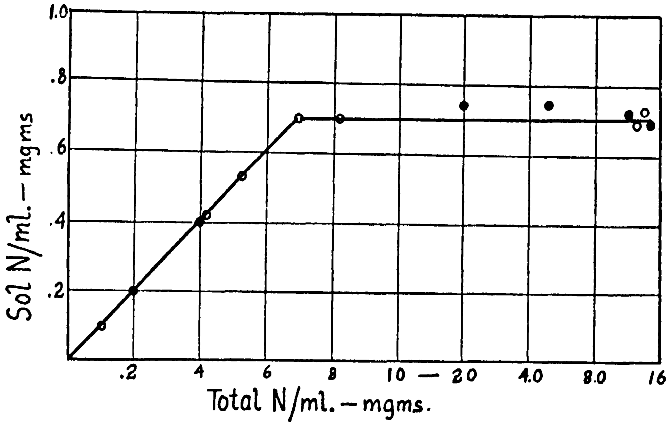

FIG. 6. Solubility of crystalline pepsin in 0.522 M sodium sulfate and 2.5 × 10^{-5} N sulfuric acid at 20°C.

TABLE VI

$$\text{Ratio}\ \frac{\text{Activity}}{\text{Nitrogen}} = [\text{PU}]^{\text{gel. V.}}_{\text{gm. N}}$$

Experiment	Pepsin	Solvent	In precipitate when nearly all soluble	In solution when large excess of solid present	In original crystals
26	Crystals	0.05 acetate pH 4.65, 0.50 sat. $MgSO_4$	0.75 1.10	1.00 1.09	1.11 ± 0.05
23	"	" " " " " " "	1.2	1.11	
22	Amorphous	" " " " " " "	1.20	1.16	
21	"	0.01 " " " " " "	1.09	1.02 1.13	
20	"	" " " " " " "	1.11	1.21	

first precipitate to appear is also the same in the experiments with amorphous and crystalline material as is shown in Table VI. If old preparations which have not been freed from the decomposition products are used, the activity-nitrogen ratio at this point will be low. An indication of this is shown in the first figure in the table and in some of the figures.

The material is therefore either a pure substance or a solid solution the components of which have nearly the same solubility in all the solvents tried.

A summary of the solubilities in the various solvents is given in Table VII.

TABLE VII

Solubility of Pepsin

Preparation	Solvent	Temperature	Solubility N/ml.
			mg.
Crystals...........	0.522 M Na_2SO_4, 2.5×10^{-5} N H_2SO_4	20°	0.70
"	0.444 M Na_2SO_4, 0.0556 acetate buffer pH 4.65	20°	.43
Amorphous.........	" " " " " " "	20°	2.2
Crystals...........	0.05 acetate pH 4.65, 0.50 sat. $MgSO_4$	8°	.22
Amorphous.........	" " " " " " "	20°	1.00
"	0.01 " " " " " "	20°	.65

Inactivation.—It was pointed out by Pekelharing that the temperature coefficient for the inactivation of enzymes and especially of pepsin was extremely high and agreed with that for the denaturization of proteins. No other reaction known has such an enormous temperature coefficient and the agreement of the two values suggests that the enzymes are proteins. Pekelharing also showed that loss of activity in strong acid solution was accompanied by the appearance of denatured, insoluble protein. It can be shown that inactivation either by heat or by alkali is quantitatively proportional to the denaturization of the protein.

Inactivation by Alkali.—It is well known that pepsin is very sensitive to alkali. Goulding, Wasteneys and Borsook (13) showed that this inactivation could be separated into two reactions, one of which was instantaneous; and further that if the amount of this inactivation were plotted against the pH a titration curve with a pK of 6.85 was obtained. These experiments were repeated and the amount of inactivation compared with the amount of denatured protein found.

Experimental.—A series of 1 per cent solutions of the pepsin was made and adjusted to about pH 5. Increasing amounts of alkali were then added to these solutions so as to give a series of solutions varying in pH from about 5.5 to 9. An amount of acid equivalent to the amount of alkali in the tube, was added immediately after the addition of the alkali and the activity of the resulting solution determined after neutralization. 5 ml. portions were added to 5 ml. of a solution of half-saturated sodium sulfate and M/500 sulfuric acid. The unchanged pepsin is soluble under these conditions (to about 1 per cent) but the denatured protein is insoluble. The denatured protein therefore precipitates and the unchanged protein nitrogen is determined from the filtrate. In this way the percentage inactivation and percentage denaturization may be determined. The denatured protein is rapidly digested by the active enzyme so these solutions must be precipitated and filtered as soon as possible.

Control experiments with known mixtures of denatured and active pepsin show that very little activity is carried down with this precipitate if the activity is determined by hydrolysis of casein. If the activity is determined by hydrolysis of gelatin, however, the precipitate of denatured protein may be quite active; and this result is evidently due to the abnormal activity observed throughout in relation to gelatin hydrolysis. This complication may be avoided by determining the activity of the solution before precipitation.

The results of such an experiment are shown in Fig. 7. The inactivation of the pepsin is quantitatively parallel with the denaturization of the protein since the percentage of activity left is the same as the percentage of soluble nitrogen remaining. The curve is very similar to that of Wasteneys and Borsook but is a little more to the alkaline side.

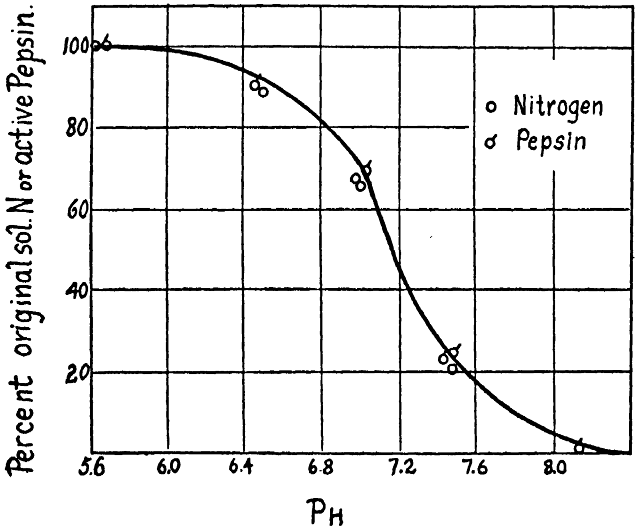

FIG. 7. Percentage inactivation and percentage denaturization of pepsin at various pH at 20°C.

Inactivation by Heat.—Pekelharing noted that when pepsin solutions were allowed to stand in a range of acidity where the pepsin is active, the protein was digested and at the same time the activity of the solution decreased. He concluded from this that the pepsin digested itself. It can easily be shown, as was mentioned above, that the denatured protein is rapidly digested and it seems probable that the effect noticed by Pekelharing was the result of denaturization of the protein followed by hydrolysis. It is then unnecessary to suppose that the enzyme digests itself. Pepsin solutions when heated slowly or when maintained at a temperature at which the inactivation proceeds slowly, do not contain denatured, insoluble protein since the latter is hydrolyzed as rapidly as it is formed. Under these conditions inactivation of pepsin is accompanied by the appearance of soluble nitrogen instead of insoluble; and it is difficult to make a sharp separation of the original pepsin from the products of hydrolysis. The result of an experiment which shows the digestion of denatured pepsin by active pepsin is given in Table VIII in which mixtures of various amounts of active and denatured pepsin were made up at pH 3 and left at 35°C. for 24 hours.

TABLE VIII

Digestion of Inactivated Pepsin pH 3.0, 35°C.

Pepsin dissolved in HCl, pH 3.0 = Solution A
50 ml. A boiled, cooled = Solution D
A and D mixed in various proportions,
2 ml. sample of mixture taken and
2 ml. M/1 Na_2SO_4 added, filtered,
N/ml. determined in filtrate.

A	20	10	5	0
D	0	10	15	20
Mg. soluble N in filtrate after:				
0 hrs.	.30	.11	.07	0
20 "	.30	.30	.28	.01

The denatured pepsin is precipitated by M/1 Na_2SO_4 at pH 3, while the active pepsin is soluble. The table shows that the inactive and insoluble pepsin is dissolved by the active pepsin.

It may be shown, however, by means of solubility experiments, that the loss of inactivation in the solution is exactly paralleled by the disappearance of the original protein. If a saturated solution of pepsin in M/300 hydrochloric acid is placed at 65°C. the activity is rapidly lost so that only about half the original activity remains after 15 minutes. No precipitate occurs but the formol titration increases. If the solution is now cooled and stirred again with a large quantity of crystalline pepsin more of the crystals must go into solution to replace the material which has been changed. If the inactivation of the enzyme is accompanied by some chemical change in the protein then the amount of material which will dissolve must be proportional to the percentage loss in activity. That is, if half the activity is lost the increase in solubility must be equal to half the original solubility (assuming the solubility to be unaffected by the presence of the inactivated enzyme). On the other hand, the activity per milliliter of the solution after being stirred with the solid should return to its original value. The result of an experiment designed to test this assumption is shown in Table IX. The solution was heated for 15 minutes at 65°C. and in that time the activity per milliliter had been reduced to 50 per cent of its original value, whereas the formol titration had increased 50 per cent and the total N per milliliter was unchanged since no precipitate appeared. The solution was then cooled and stirred at 20°C. with more crystals. The suspension was then filtered and the supernatant analyzed. The activity per milliliter is now the same as that of the original solution while the nitrogen per milliliter is 50 per cent higher than the original value. The formol titration has been increased by 50 per cent of its original value also. The results show that the loss of activity is exactly paralleled by some chemical change in the protein molecule.

TABLE IX

Inactivated Pepsin and Solubility. 65°C.

	$[PU]^{gel. V.}_{ml.} \times 10^3$	N/ml.	Formol/ml.
		mg.	*ml. 0.01 NaOH*
M/300 HCl, stirred 1 hr. with pepsin, 20°C. Centrifuge	0.315	0.30	0.22
Supernatant heated at 65°C. for 15 min.	.160	.30	.33
Pepsin crystals added and stir 1 hr. Centrifuge			
Observed	.325	.45	.45
Calculated	.315	.455	.44

The Rate of Inactivation at 65°C.—It has frequently been found that the rate of inactivation of enzymes does not follow the course of a simple monomolecular reaction but becomes slower as the reaction proceeds. It was found by the writer (6) that this peculiarity was connected with the purity of the enzyme solution and that the rate of inactivation of more highly purified solutions agreed with the monomolecular reaction rate, while those solutions containing inhibiting substances did not. On this basis the rate of inactivation of the crystalline material would be expected to be monomolecular. The result of an experiment in which the rate of inactivation of a 0.06 per cent solution of pepsin in 0.001 M hydrochloric acid (pH 3) at 65°C. is shown in Fig. 8. The reaction follows the theoretical monomolecular course quite closely.

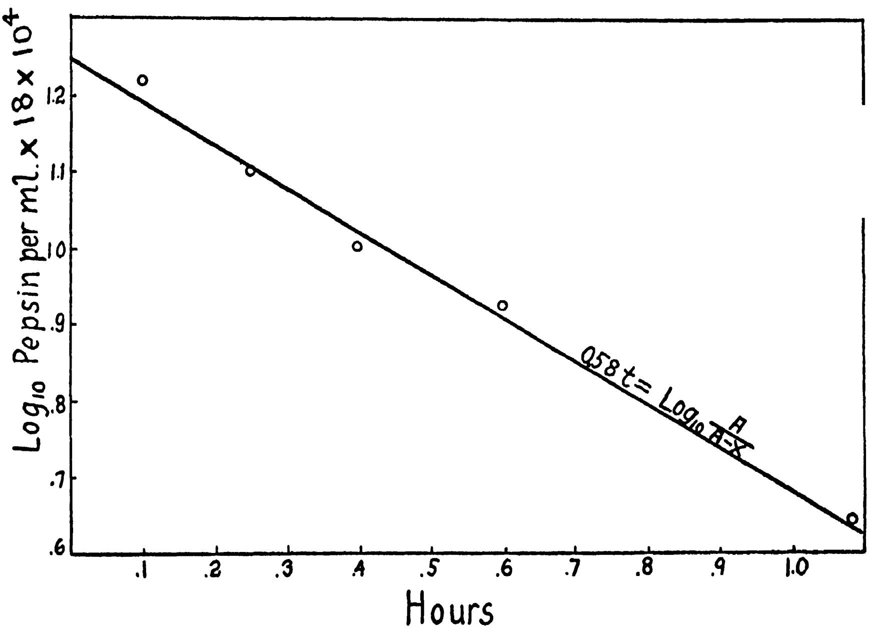

FIG. 8. Rate of inactivation of 0.06 per cent pepsin in 0.001 M hydrochloric acid at 65°C.

Identity of the Diffusion Coefficient of the Protein and of the Active Material.—The preceding experiments have shown that the activity cannot be separated from the protein molecule either by solubility measurements or inactivation experiments. It might be assumed, however, that the crystalline substance was analogous to a double salt and that the active material and the protein were in equilibrium with each other but were separate in solution. If this were the case it would be expected that the rate of diffusion of the two would be different. The diffusion coefficients were therefore determined by the method previously described (14). The experiments were carried out at 8°C. and the amount of material diffusing was determined by analyzing for nitrogen and also by activity determinations. The results of this experiment are shown in Table X. The diffusion coefficient was found to be the same when determined by either nitrogen or by activity. The radius (and presumably the weight) of the molecule containing the nitrogen and of the active molecule must therefore be the same, so that there is no indication that the protein molecule is different from the molecule responsible for the activity even in solution. The diffusion coefficient is found to be 0.048 ± 0.0005 cm.^2 per day which, according to Einstein's formula, gives a molecular weight of 36,000. This figure agrees very well with the molecular weight of 37,000 as determined by osmotic pressure measurements. The agreement between these two figures shows that the molecules must be nearly all the same size since the molecular weight gives a mean value while the diffusion experiment as carried out gives a minimum value. The agreement also shows that the molecule is not hydrated to any great extent, since the value used for the specific gravity in calculating the molecular weight from the diffusion coefficient is the specific gravity of dry protein; and the value for the molecular weight so obtained agrees with that found by osmotic pressure.[3]

TABLE X

Diffusion Coefficient, Radius of Molecule and Molecular Weight

8°C.	1 per cent solution in M/1 acetate buffer pH 4.6, viscosity of buffer = 0.017.		
D	from activity determination	=	.047 ± .0005 cm.^2/day
	from N determination	=	.0468 ± .0005 cm.^2/day
		=	5.44×10^{-7} cm.^2/sec.
r	= 2.22×10^{-7} cm.		
	= 2.22 mμ		
M	= 37,000 ± 1,000		

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[3] Preliminary experiments (15) gave larger diffusion coefficients corresponding to a molecular weight of about 10,000, but this result was found to be due to an error in calibrating the diffusion cell.

Antipepsin.—If the enzyme is a protein an antibody should be formed on injection into an animal. The production of such antienzymes has been reported in the literature but the results are uncertain, as Euler has pointed out (16), because of lack of information in regard to the activity of the preparation used. In order to see if any antibody could be produced and if its production were associated with the activity of the preparation, two rabbits were immunized, one with an active preparation of the enzyme and one with a preparation of the same concentration which had been inactivated by alkali. 5 ml. injections of a 1 per cent solution of the active or inactive material were made intraperitoneally at intervals of about 1 week. Four injections were made; the rabbits were bled 2 weeks after the final injection and the serum obtained in the usual way. These sera gave a precipitin reaction with the pepsin solution, as shown in Table XI. Normal serum gave no precipitate with either the active or the inactive pepsin preparation. The serum obtained from the rabbit injected with active pepsin gave a positive precipitin reaction in a dilution of about 1/2,000 with the active pepsin solution and to about 1/16 with the inactive solution. The serum from the rabbit immunized with denatured pepsin gave a precipitin test with the denatured preparation in a dilution of about 1/8 per cent solution and with the active preparation to

TABLE XI

Antiserum

1 ml. 1 per cent pepsin solution in M/5 4.6 acetate buffer, diluted with buffer, + 0.15 ml. serum.

Pepsin dilution		1	1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1,000	1/2,000	0
Pepsin	Serum	0 serum	.15 ml. serum												
Active	Active	–	++	++	++	++	++	+	+	+	+	+	+	+	–
"	Denatured	–	++	++	++	++	+	–	–	–	–	–	–	–	–
"	Normal	–	–	–	–	–	–	–	–						
Denatured	Active	–	++	++	+	+	+	–	–	–	–	–	–	–	–
"	Denatured	–	++	++	++	+	–	–	–	–	–	–	–	–	–
"	Normal	–	–	–	–	–	–	–	–	–	–	–	–	–	–

about the same extent. Both sera gave precipitin reactions with a 1 per cent solution of pepsin inactivated by boiling in a dilution of about 1/16. As might be expected, therefore, the sera were not strictly specific but gave cross precipitations with both active and denatured pepsin. The inhibiting effect of the sera on the activity was tested by mixing increasing amounts of the antiserum with a small amount of active pepsin and determining the rate of digestion of casein or gelatin with this mixture. The result of this experiment is shown in Table XII.

TABLE XII

Effect of Antiserum on Digestion of Casein or Gelatin by Pepsin

Serum diluted as noted + M/5 4.6 acetate buffer. Pepsin in 4.6 acetate buffer. 1 ml. pepsin solution + 1 ml. serum dilution.

Gelatin

	Final dilution of serum					
Serum	1/4	1/8	1/16	1/32	1/64	0
	$[PU]^{gel.\ V.}_{ml.}$ after 3 hrs. × 10^3					
Normal	0.075	0.10	0.11	0.12	0.11	0.12
Antidenatured	.060	.10	.09	.10	.11	.12
Antiactive	.045	.075	.10	.10	.11	.12

Casein

Serum	0	Normal	Anti-denatured	Antiactive
Soluble N in filtrate, mg	2.2	2.0	1.87	1.87
$\approx[PU]^{cas.\ S.}_{ml.}$	.015	.008	.007	.007

Both the serum prepared by the injection of the active pepsin and that prepared by the injection of the denatured pepsin inhibit the action of the enzyme to about the same extent when tested by the solution of casein (cas. S. method). The normal serum inactivates somewhat less although there is no very great difference. It is difficult to compare the action of the various sera quantitatively from this experiment. The effect may be seen more clearly by determining the rate of hydrolysis of gelatin (gel. V. method) with a small amount of pepsin to which has been added increasing amounts of the various sera. The result of such an experiment is also shown in Table XII. The active serum inhibits quite strongly in a dilution of 1:4, the denatured serum less so and the normal serum still less. This inhibiting effect is evidently very weak compared with that of many antitoxins, although the precipitin reaction is about what would be expected. This may be due to the fact that the pepsin injected must be almost instantly inactivated since, as was shown above, the pepsin is instantly more than half inactivated at a pH of 7. It is probable therefore that the active pepsin is in the circulation of the animal for only a very short time.

The writer is indebted to Dr. F. S. Jones and Dr. A. P. Krueger for advice and assistance in connection with these experiments.

VI

Conclusions as to the Purity of the Preparation

The preceding experiments have shown that no evidence for the existence of a mixture of active and inactive material in the crystals could be obtained by recrystallization, solubility determinations in a series of solvents, inactivation by either heat or alkali, or by the rate of diffusion. It is reasonable to conclude therefore that the material is either a pure substance or a solid solution of two very closely related substances. If it is a solid solution of two or more substances it must be further assumed that these substances have about the same degree of solubility in the various solvents used, as well as the same diffusion coefficient and rate of inactivation or denaturization by heat. It must also be assumed that both substances are changed by alkali at the same rate and to the same extent. This could hardly be true with the possible exception of two closely related proteins. It is conceivable that two proteins might be indistinguishable by any of the tests applied in this work. But in this case it would follow that the enzyme itself was a protein and this, after all, is the main point. It does not necessarily follow even if the material represents the pure enzyme that it is the most active preparation that can be obtained nor that it is the only compound which has proteolytic activity. There is some evidence that the activity of the preparation may depend on its physical state as is known to be the case with the catalytic activity of colloidal metals. It is possible, on the other hand, that hemoglobin is the type structure for the enzymes and that they consist of an active group combined with a protein as suggested by Pekelharing. The active group may be too unstable to exist alone, but it is quite conceivable that a series of compounds may exist containing varying numbers of active groups combined with the protein, and that the activity of the compound would depend on the number of these active groups. This hypothetical complex would not differ much from that assumed by Willstätter (18) and his coworkers, except that it supposes a definite chemical compound with the protective group in place of an adsorption complex. It is of course possible that both types of complex may be formed under suitable conditions. The reactivation of enzymes as reported in the literature also suggests their protein nature since the conditions for this reactivation are similar to those found by Anson and Mirsky (20) to be suitable for the formation of native from denatured protein. The fact that the crystalline urease prepared by Sumner is also a protein and that the temperature coefficient for the rate of inactivation of enzymes in general is that characteristic for the denaturization of proteins, suggests that the protein fraction in the purification of enzymes be given special attention even though it may not be the most active fraction.

The crystalline pepsin resembles the amorphous preparations obtained previously by Pekelharing, Ringer, Fenger, Andrew and Ralston and other workers. It is probable that the preparations obtained by these workers were nearly pure pepsin. Both Pekelharing and Ringer (17) obtained preparations free from phosphorus so that there may be several proteolytically active forms.

Pekelharing showed that the same protein material could be obtained from gastric juice as from autolyzed gastric mucosa so that it is probable that the crystalline material could also be readily prepared from gastric juice. It seems fair to conclude therefore that the crystalline protein described in this paper is identical with the enzyme pepsin as secreted by the animal.

VII

Proteolysis Is a Homogeneous Reaction

Sørensen has shown that a solution of egg albumin is a one-phase system and the solubility measurements described in this paper show that a solution of pepsin consists also of only one phase. The reaction between dissolved egg albumin and dissolved pepsin therefore is a case of homogeneous, rather than heterogeneous catalysis.

SUMMARY

A method is described for the preparation of a crystalline protein from commercial pepsin preparations which has powerful peptic activity. The composition, optical activity, and proteolytic activity of this protein remain constant through seven successive crystallizations. No evidence for the presence of a mixture or of a solid solution is found in a study of the solubility of the protein in a series of different salt solutions, nor from the diffusion coefficient or from the rate of inactivation. These results indicate that the material is a pure substance or possibly a solid solution of two or more substances having nearly the same solubility in all the various solvents studied. It seems reasonable to conclude from these experiments that the possibility of a mixture must be limited to a mixture of proteins, so that the conclusion seems justified that pepsin itself is a protein.

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CRYSTALLINE PEPSIN

II. GENERAL PROPERTIES AND EXPERIMENTAL METHODS

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The isolation of a crystalline protein having proteolytic activity was described in the preceding paper (1). The results of solubility, inactivation, and diffusion experiments showed that there was reason to believe that the preparation was a pure substance and that the peptic activity was a property of the protein molecule itself. The following paper contains some further properties of the pepsin and a description of the experimental procedure.

Solubility in Various Concentrations of Sodium Sulfate.—The solubility in various concentrations of sodium sulfate is shown graphically in Fig. 1. A concentrated suspension of the crystalline material was made in sodium sulfate of the required concentration made up in 0.001 M sulfuric acid. These suspensions were stirred at 35°C. for 10 minutes and then cooled to 25°C. for 24 hours. The solubility was therefore reached from the supersaturated side. There is a sharp maximum of solubility at about 0.1 M sodium sulfate.

Solubility at Different Temperatures.—The solubility in 0.50 saturated $MgSO_4$ and 0.05 acetate pH 4.65 at various temperatures is shown in Fig. 2. The solubility increases slightly but there are no sharp breaks in the curve and therefore no indication that the material is a double salt, nor that different solid phases appear at different temperatures.

Solubility in Various Concentrations of Hydrochloric or Sulfuric Acid.—These determinations were made by stirring a concentrated suspension with various concentrations of acid as described for the sodium sulfate experiments. The results are given in Fig. 3. There is a minimum of solubility at about pH 2.75 corresponding presumably to the isoelectric point. The solubility increases rapidly on both sides of this point. The increase in solubility on the acid side cannot be ascribed to salt effect since the series represented by triangles was made with a mixture of hydrochloric acid and sodium chloride in which the

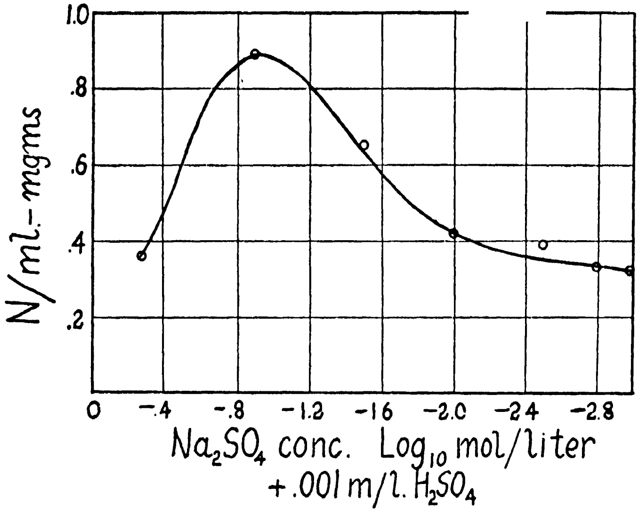

FIG. 1. Solubility of crystalline pepsin in various concentrations of sodium sulfate made up in 0.001 M hydrochloric acid at 25°C.

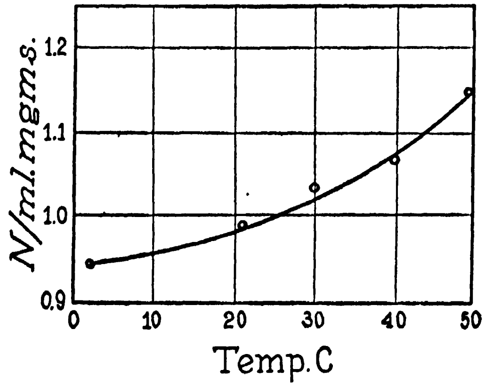

FIG. 2. Solubility of crystalline pepsin in 0.5 saturated magnesium sulfate and 0.05 M acetate at various temperatures.

chloride concentration was kept constant throughout. When the solubility is plotted against the concentration of acid instead of the pH the solubility increases in direct proportion to the addition of acid and each millimole of acid dissolves about 0.05 mg. of nitrogen, corresponding to 0.33 mg. of pepsin. This is the expected relation for a monovalent base (2). On the alkaline side of the minimum the solubility curve is convex downward. This is the theoretical shape for the solubility curve of a polyvalent acid.

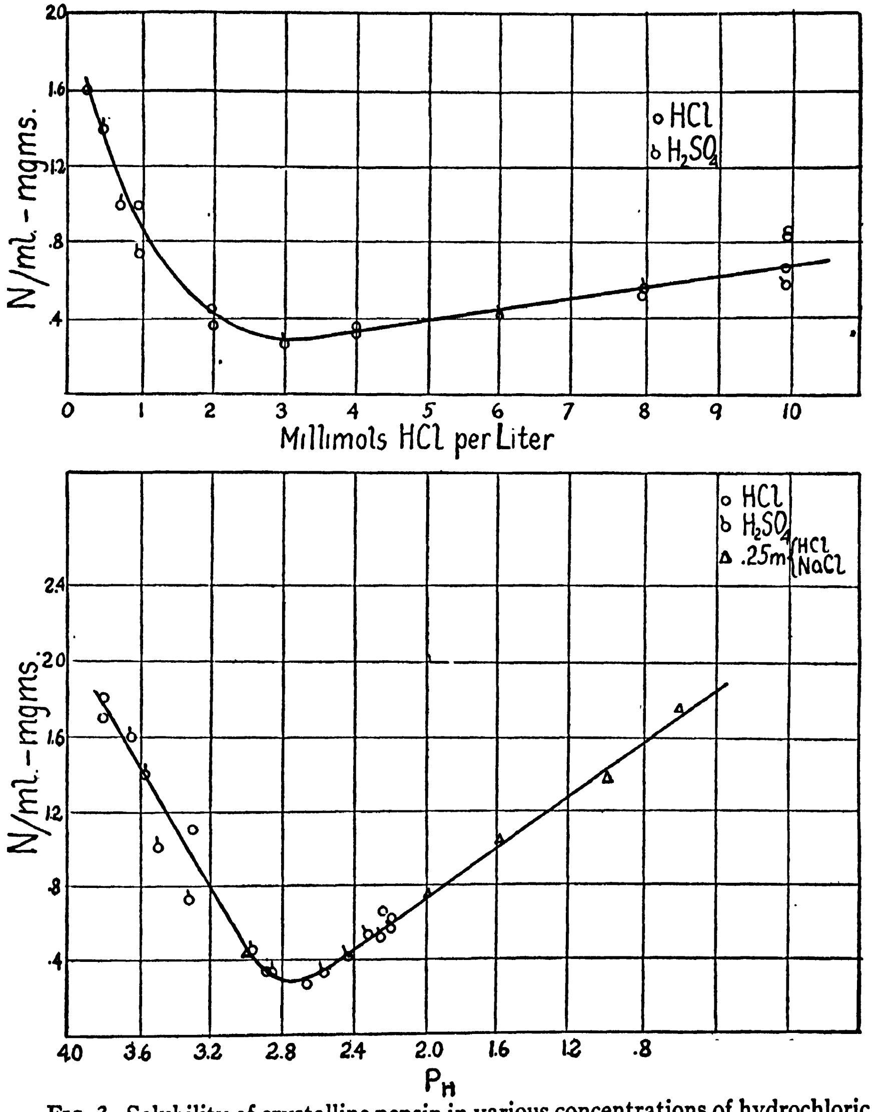

FIG. 3. Solubility of crystalline pepsin in various concentrations of hydrochloric or sulfuric acid.

Titration Curve.—The titration curve of the pepsin is shown in Fig. 4. Owing to its insolubility the curve could not be followed on the acid side; but apparently there must be one or more groups with a pK of 1 or less. The curve again indicates an isoelectric point of pH 2.75. Some titrations were made in the presence of solid and indicated that the solid phase appearing on the alkaline side is a salt of the pepsin and acid.

The writer is indebted to Dr. H. S. Simms for the determination of the titration curve.

The Isoelectric Point as Determined by Migration Experiments.—The solubility and titration curves indicate an isoelectric point at about pH 2.75. This was confirmed by measurements of the migration of finely ground crystals at various pH as determined microscopically (3). No migration occurred at pH 3. The crystals migrated to the anode on the alkaline side of 3 and to the cathode on the acid side of 3. The isoelectric point of the preparation is therefore probably 2.75, which is not far from that found by Michaelis and Davidson (4) in a study of the migration of crude pepsin preparations. Ringer (5) had, however, stated that some pepsin preparations were always negatively charged and the writer (6) had found that the distribution of pepsin with gelatin particles also indicated that the enzyme was always negatively

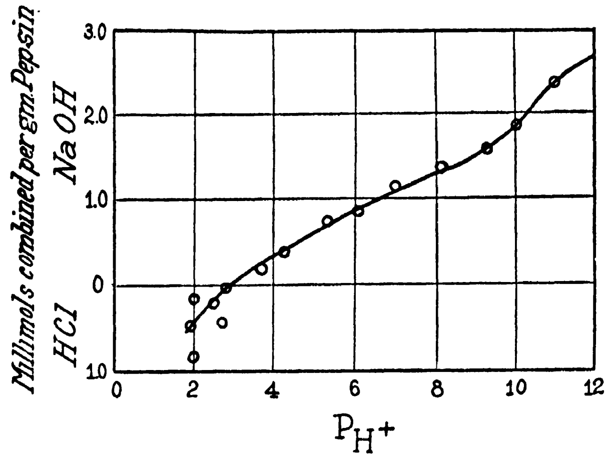

FIG. 4. Titration curve of crystalline pepsin at 22°C. with hydrochloric acid and sodium hydroxide.

charged on the alkaline side of pH 2.0. It does not seem possible at present to account for these discrepancies.

Molecular Weight.—The molecular weight of the pepsin was determined by osmotic pressure measurements in molar sodium acetate buffer at pH 4.60. Since this is quite far from the isoelectric point it is necessary to show that the observed pressure is due to the protein itself and not to a Donnan equilibrium. The effect of the Donnan equilibrium would be slight in molar salt solution and would be expected to increase as the concentration of the protein increased. The experiments, however, show that the calculated molecular weight was constant within the experimental error for a 4 per cent and a 2 per cent solution. There was probably therefore very little Donnan effect. The molecular weight may also be calculated from the diffusion coefficient by means of Einstein's formula, and also from the percentage of phosphorus, chlorine and sulfur. The results of these calculations are shown in Table I. The various methods all give values for the molecular weight between 33,000 and 38,000.

TABLE I

Molecular Weight

Method	Temperature	Concentration of solution	Solvent	M
Osmotic pressure	8°	4 per cent	M/1 acetate pH 4.6	34,000
			" " "	33,000
	8°	2 " "	" " "	36,000
Diffusion coefficient				
Activity	8°	1 " "	" " "	37,000 ± 1,000
Nitrogen			" " "	36,000 ± 1,000
From P content	1 atom P per mole			40,000
Cl "	2 atoms Cl " "			35,000
S "	10 " S " "			36,000

Experimental Procedure

Material.—The starting point of this work was Parke, Davis pepsin U. S. P. 1:10,000. This material has already been highly concentrated from autolyzed pig stomachs. According to information kindly furnished by Mr. F. O. Taylor, Chief

Chemist of Parke, Davis and Company, the method of purification is in general that of Davis and Merker (7).

Rate of Hydrolysis of Casein, Egg Albumin, Edestin or Gelatin as Determined by the Increase in Carboxyl Groups

The egg albumin had been crystallized three times. Hammersten's casein (Kahlbaum) was used. The edestin was prepared from hemp seed and had been recrystallized twice. The gelatin was prepared from Cooper's powdered gelatin as previously described (8).

The protein solutions were titrated to the required pH with HCl and diluted so as to contain 5 gm. protein per 100 ml. They were then heated to boiling to denature the proteins and stored at 5°C. 5 ml. of the protein solution are pipetted into a series of 50 ml. centrifuge tubes and placed in a water bath at 35.5°C. 1 ml. of the pepsin solution is added to each tube. After about 2, 4, 6 and 8 minutes 5 ml. of 0.10 NaOH and 1 ml. of formalin are added to each tube. They are then titrated to pH 10 with N/50 NaOH (9). The increase in the alkali needed over that used for a control tube containing 1 ml. boiled pepsin solution is the titration. These figures are then plotted against the time of hydrolysis and the increase in the formol titration for the first minute interpolated from the curve. The proteolytic activity per gram is equal to this amount, expressed as milliequivalents divided by the grams dry weight per milliliter of the pepsin solution used.

$$i.e., \; [\mathrm{PU}]_{\mathrm{gm.}} = \frac{\text{ml. N/50 NaOH} \times 20 \text{ per minute}}{\text{mg. pepsin per ml.}}$$

The determination is quite accurate with casein or edestin but in the case of egg albumin and gelatin the rate of hydrolysis decreases rapidly after an increase of only 0.3 to 0.5 ml. N/50 NaOH, so that interpolation is difficult unless very small titrations are used and in this case the experimental error of the titration is large.

Determination of Activity by Comparative Methods

The activity determined in this way probably represents the most rational measure of the rate of hydrolysis. For routine comparative purposes, however, it is not convenient since it requires a number of analyses for each determination and since the experimental error is rather large. After the activity in rational units has been determined by this method, however, it is possible to use comparative methods which are more accurate and also more convenient. In order to do this a solution the activity of which has been determined by the previous method is used as a standard. Several comparative methods of this kind have been used.

Increase in Formol Titration after 1 Hour at 35°C. (cas. F.), (gel. F.), etc.—Increasing amounts of the standard pepsin solution containing a known number of PU as determined by the rate of liberation of carboxyl groups as described above, are added to 5 ml. portions of the various protein solutions and the hydrolysis allowed to proceed for 1 hour at 35.5°C. At the end of this time the increase in the formol titration is determined as described above. This increase is then plotted against the number of proteolytic units added so as to give a standard curve. The activity of an unknown solution is then determined by carrying out the hydrolysis in the same way and interpolating the value of the activity from the standard curve.

Solution of Casein (*cas. S.*).—1 ml. of various dilutions of the standard pepsin solution is added to 5 ml. of 5 per cent casein pH 2 and kept at 35.5°C. for 21 hours. 5 ml. of this solution is then pipetted into 5 ml. of 20 per cent trichloracetic acid, filtered and the total nitrogen determined in 5 ml. of the filtrate. A standard curve is plotted from these figures and the activity of the unknown solution found by interpolation from this curve. This is the most accurate method, the probable error of a single determination being less than 2 per cent.

Rennet Action (*R*).—The amount of pepsin solution which is just sufficient to solidify 5 ml. of milk at 35.5°C. in 2 hours is determined. This figure cannot be compared directly with the activity as determined by the liberation of carboxyl groups. For purposes of comparison therefore it is arbitrarily assumed that the activity of the standard solution of crystalline pepsin is the same with respect to rennet action as with respect to the hydrolysis of casein as determined by the rate of liberation of carboxyl groups. 0.085 mg. crystalline pepsin equivalent to 0.008 $[\mathrm{PU}]^{\mathrm{cas.\,F.}}$ coagulates 5 ml. of milk under the above conditions. 1 ml. of a solution which causes coagulation of 5 ml. of milk in 2 hours therefore contains 0.008 $[\mathrm{PU}]^{\mathrm{R}}$. The time varies greatly with different samples of milk so that it is necessary to run a known solution simultaneously with the unknown.

Gelatin Liquefaction Method (*10*) *pH 5* (*gel. V.*).—0.2 ml. of the standard pepsin solution is added to 5 ml. of 2.5 per cent solution of isoelectric gelatin in water and the mixture poured into a viscosimeter at 35.5°C. The change in viscosity is then determined at intervals and the time of outflow after various time intervals plotted against the elapsed time since mixing. The time required to cause a 3 per cent change in the original viscosity is then interpolated from these curves. The time required to cause this change is inversely proportional to the quantity of pepsin so that the activity of an unknown solution in terms of the standard is found by proportion. Since there is no simple relation between the rate of change in viscosity and the liberation of carboxyl groups (11), it is assumed arbitrarily that the activity of the standard crystalline pepsin solution is that determined by the rate of liberation of carboxyl groups from pH 2.5 gelatin. Under these conditions 0.14 mg. of crystalline pepsin equivalent to 2.4×10^{-5} $[\mathrm{PU}]^{\mathrm{gel.\,F.}}$ cause a 3 per cent change of viscosity in 0.30 hours. Since the activity is inversely proportional to the time

$$[\mathrm{PU}]^{\mathrm{gel.\,V.}}_{0.2\ \mathrm{ml.}} = \frac{0.72 \times 10^{-5}}{t\ \mathrm{hrs.}}$$

and

$$[\mathrm{PU}]_{\mathrm{ml.}}^{\mathrm{gel\ V.}} = \frac{3.6 \times 10^{-5}}{t \text{ hrs.}}$$

where t is the time in hours for 0.2 ml. of the unknown solution to cause a change of 3 per cent in the viscosity of the standard gelatin solution.

Nitrogen Determination.—The nitrogen was determined by a slight modification of the Folin and Farmer (12) micromethod using the following digestion mixture:

2 ml. concentrated H_2SO_4
1.5 gm. K_2SO_4
0.2 gm. mercuric oxide

Digestion is continued 20 minutes after the solution is colorless in 50 ml. Kjeldahl flasks having a constriction half-way up the neck to prevent loss of acid.

35 ml. H_2O, 0.2 gm. sodium hypophosphite and 10 ml. of saturated NaOH are added and the solution distilled through two Kjeldahl traps into standard N/50 acid. 2 to 5 mg. of nitrogen are taken for a determination. The method is accurate to about 0.2 per cent provided the double trap is used. Otherwise there are irregular variations due to spattering of the alkali.

P Determination.—The excellent method described by M. Sørensen (13) was used.

Experimental Methods for the Solubility Determinations

Crystals.—The first attempts to determine the solubility of pepsin were made in the usual way by stirring a suspension of the solid with the liquid and analyzing the supernatant fluid after various times. It was found, however, that equilibrium could not be obtained in this way except when a very large amount of the solid was present. Otherwise, the amount of material dissolved increased rapidly for the first 24 hours and gave what appeared to be an equilibrium value. This value, however, was different with different quantities of precipitate, being higher for the larger quantity. If the stirring were continued, however, it was found that the solubility continued to increase slowly in all the suspensions and this increase continued at a nearly constant rate, apparently indefinitely. At the same time the ratio of activity to nitrogen in the filtrates decreased and a form of nitrogen which could not be coagulated by heat began to appear in the solution. It was evident therefore that this slowly continued increase in soluble protein was not simply a process of solution but was due to the decompostition of the protein. Various methods of stirring combined with low temperatures were tried without success, although it was found that stirring with a glass ball in a test tube completely filled with solution gave much better results.

With large quantities of precipitate constant values for the solubility could be obtained very rapidly by stirring the suspension in a long narrow tube, the stirrer consisting of a glass rod of about half the diameter of the tube so that there is only a thin layer of suspension between the stirrer and the walls of the tube. The stirrer was rotated at about 500 R.P.M. There is very efficient stirring under these conditions and constant values for the solubility were obtained after about 10 minutes, provided the amount of solid material was more than 3 or 4 times as much as the quantity in solution. The figure for the solubility under these conditions was found to be independent of the quantity of precipitate when relatively large amounts of precipitate were used. However, if the quantity of precipitate were small then equilibrium values could not be obtained before decomposition commenced, so that it was not possible in this way to obtain the part of the curve where there is very little solid. This portion of the curve, however, could be obtained from the supersaturated side. The method finally adopted for solubility measurements may be outlined as follows.

A suspension of freshly recrystallized protein was filtered by suction and washed on the filter 5 or 6 times with the solvent that was to be used for the solubility determinations. About 25 gm. of the filter cake were then suspended in 100 ml. of the solvent and stirred for 15 minutes at the desired temperature, the stirrer being arranged as described above. The suspension was then filtered and the nitrogen content of the filtrate determined. The precipitate was stirred again with 100 ml. of the solvent and this process was repeated until successive determinations gave the same value for the nitrogen in the filtrate. Aliquot portions of the suspension were diluted with solvent so as to give a series of suspensions containing 1/2, 1/4, 1/8 and 1/16 of the concentration of solid present in the original suspension. Each of these suspensions was then stirred for 15 to 20 minutes, filtered and the nitrogen, proteolytic activity and optical rotation of the filtrate determined. These figures represent the solubility as determined from the undersaturated side.

A second series of suspensions was made up in the same way and stirred at 45°C. for 5 to 10 minutes. A small sample of supernatant was taken at 45°C. and analyzed for nitrogen. The figure obtained in this way was always higher than that found at the lower temperature. These suspensions were then cooled to the original temperature and stirred occasionally. After 24 hours they were filtered and the supernatant analyzed as in the first series. This method gives the solubility figure from the supersaturated side. In this way the solubility could be determined for suspensions containing more than twice as much solid protein as dissolved protein. In this range the solubilities determined from the undersaturated side nearly always agreed with those from the supersaturated side showing that they were real equilibrium values. When less solid was present, however, this was not the case. The values from the supersaturated side were always higher than those from the undersaturated side and were frequently higher than those obtained with large quantities of solids from the supersaturated side. In other words, they showed that equilibrium was not obtained when only a small quantity of solid was present. Attempts to obtain equilibrium values by longer stirring resulted only in indefinite increase in solubility accompanied with inactivation. The solubilities at the point where very little solid was present could be obtained, however, by diluting a supersaturated solution and then inoculating with small amounts of solid. About 50 ml. of the original heavy suspension were stirred at 45°C. and filtered at this temperature. The filtrate was then diluted so as to give a series of solutions containing from 0.5 mg. to 3 or 4 mg. of nitrogen per milliliter. A few drops of the heavy suspension were then added to this series of solutions and the solutions cooled to the original temperature. In those solutions containing less than the saturation concentration the added solid dissolved and the solution became perfectly clear. The more concentrated ones remained cloudy and in those which had been diluted very little more solid crystallized out. These solutions were filtered and analyzed after 24 hours in the same way as the other series.

In this way a series of points were obtained showing the solubility up to and just beyond the appearance of the solid phase. The values were reproducible and those obtained when very little solid was present usually agreed well with the values obtained by the methods described for the case when a large amount of solid was present. There still remains a very small portion of the solubility-concentration curve corresponding to the point where the amount of solid was about equal to the amount of material in solution, for which equilibrium values could not be obtained. Values obtained by inoculating the supersaturated solution were usually higher than those found for the rest of the curve. The values obtained from the undersaturated side were usually lower.

Amorphous.—The solubility of the amorphous material as obtained from the undersaturated side was determined in the same way as with the crystals by stirring the precipitate with the solvent. In order to obtain the value from the supersaturated side, however, it was necessary to dissolve the precipitate and then reprecipitate it instead of allowing a supersaturated solution to cool since under these latter conditions crystals appeared. The solvent solution was therefore made up in two parts, in one of which the amorphous material was readily soluble. 20 to 30 gm. of the washed crystals were dissolved in as small a volume as possible of this part of the desired solvent and then precipitated by addition of the other part of the solvent. The solution was stirred during the precipitation and the precipitating solution run in slowly so as to avoid local excess. The amorphous precipitate obtained in this way was redissolved in the first part of the solvent and precipitated again.

This procedure was repeated until the nitrogen content of two or more successive filtrates was the same. This was taken as the value for the solubility from the supersaturated side. The suspension was then filtered and aliquot portions of the precipitate stirred with about 10 ml. of the complete solvent for 10 to 15 minutes. These suspensions were then filtered and analyzed as with the crystalline material. In this way a series of points were obtained showing the solubility obtained with varying amounts of precipitate from the undersaturated side. In order to obtain the value from the supersaturated side with different amounts of solid present the remainder of the precipitate was dissolved in varying amounts of the first half of the solvent, so as to give a series of solutions containing from 1 to 3 or 4 mg. of nitrogen per milliliter. The second half of the solvent was then added to this series while the solution was being stirred and the suspension filtered at once. If the solutions were filtered immediately these values agreed well with those from the undersaturated side, but if the solutions were allowed to stand the values were consistently lower in those solutions in which only a small amount of solid appeared. This was at first very puzzling and it was thought that it might represent an actual fractionation of the material. However, the properties of the precipitate obtained in this way were precisely the same as that of the original material so that there was no evidence for any fractionation. Microscopic examination of these precipitates, however, showed that they contained some crystalline material, and since the solubility of the crystalline material is much less than that of the amorphous this was evidently the explanation for the low solubility obtained under these conditions. When a large amount of amorphous precipitate is present the material crystallizing out would be immediately replaced by solution of more amorphous so that under these conditions the saturation value of the amorphous precipitate was obtained. When only a small amount of amorphous material was present, however, the loss of material due to crystallization could not be replaced rapidly enough by solution of the amorphous material and so low values for the solubility resulted. In confirmation of this it was found that the values for the solubility obtained by filtering those solutions having very little precipitate immediately after the appearance of the precipitate agreed with those obtained in solutions containing larger amounts of precipitate. It was also found that inoculation of the solutions with a few crystals gave still lower values for the solubility since in this case crystallization proceeded more rapidly.

Composition of the Solutions Used.—In order to carry out the precipitation of the amorphous material as described above, it is necessary to make up the complete solvent by adding together two different solutions. Measured volumes of the two separate parts of the solvent must be used instead of making the mixture up to a constant volume in order to avoid the difficulty of correcting for the volume of the original portion of the concentrated suspension taken. The composition of the solvent is therefore expressed as the ratio of the volume of one portion to the total volume assuming that the volumes are additive. The value given in this way does not correspond strictly with the actual composition of the final solution but does define the composition of the complete solvent. For instance, a solvent designated as half-saturated magnesium sulfate and 0.05 M acetate buffer pH 4.65 refers to a solution prepared by mixing together equal volumes of saturated magnesium sulfate and 0.10 M acetate buffer pH 4.65. The saturated magnesium sulfate was prepared by stirring crystalline magnesium sulfate with water at about 18°C. The supernatant was filtered off and found to have a specific gravity at 22°C. of 1.2955 compared to water at that temperature. The sodium sulfate solution had a specific gravity at 22°C. of 1.120 and was 1.104 molar with respect to Na_2SO_4.

TABLE II

Summary of Properties of Pepsin

Crystalline form. Dihexagonal bipyramids, colorless, 0.01 to 0.1 mm. long. Positive double refraction.
Density. 1.32 gm. per ml.

$[\alpha]_D^{24°}$ (pH 4–5) −70°

Analysis. Per cent of dry weight.	Total N	Amino N	C	H	Cl	S	P	Ash	$SO_4^=$
	15.3	.80	52.4	6.67	.22	.86	.078	.40	00

Xanthoproteic and Biuret test—positive
Molisch test —negative

Diffusion coefficient 8° {M/1 acetate buffer, pH 4.6, Viscosity .017}
.047 ± .0005 cm.²/day (From N determination)
.0468 + .0005 " " (" activity determination)

Molecular weight from osmotic pressure (M/1 acetate pH 4.6)	35,000 ± 1,000
From diffusion coefficient	36,000 ± 1,000
From P content, 1 atom P per mole	40,000
Cl " 2 atoms Cl per mole	35,000
S " 10 " S " "	36,000

TABLE II—*Concluded*

Solubility	Solvent	.002 HCl	.522 M Na_2SO_4 2.5×10^{-5} N H_2SO_4	.444 M Na_2SO_4 .055 acetate pH 4.65	0 5 sat. $MgSO_4$ .05 acetate pH 4.65	0.5 sat. $MgSO_4$ .01 acetate pH 4.65
Mg. N/ml.	Crystalline	.3 (20°)	.70 (20°)	.43 (20°)	.22 (8°)	
	Amorphous			2.2 (20°)	1.00 (20°)	.65

Velocity constant inactivation
65°— pH 3.0 0.58 Time in hrs. Log_{10}.
1.98 per cent solution in HCl

Activity		
Substrate	pH	Equivalents per mole pepsin per min. 35.5°C.
Edestin	2.0	1,000
Casein	2.0	500
Denatured egg albumin	2.2	320
Gelatin	2.5	6.0

The acetate buffers were made up according to Walpole's curve (14). The pH values given are for solutions corresponding to 0.2 M acetate buffer and do not represent actual pH values of the final solution.

The analytical work and the preparation of most of the crystalline pepsin was carried out by Mr. N. Wuest.

SUMMARY

A number of properties of crystalline pepsin have been determined and are summarized in Table II.

The experimental procedure used is described.

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THE CELL SAP OF HALICYSTIS

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I

Knowledge of the chemical constitution of the vacuolar sap of *Halicystis*, found in Bermuda, has been based on the analyses by Dorcas,[1] which were performed with sap extracted from cells floated in upon the beaches.[2] During the past 5 years, samples of sap have several times been extracted from smaller cells of *Halicystis* at Bermuda, detached by hand from the substratum upon which they were growing. The quantity of this sap was not sufficient for complete analyses, but the ratio of potassium to sodium was in every case found to be very low, and in good agreement with that reported in the original analysis. This resemblance has been noted in a paper[3] dealing with the potential differences of *Halicystis* cells collected from their position of growth in 1929.

A more complete analysis is now presented, based on an adequate sample of sap from growing cells.

The material was collected in April, 1930, the temperature of the water being 19°C. The cells grow on crusts of *Lithothamnion* just below low tide, often occurring in colonies up to several hundred, and range from less than 1 mm. to more than 12 mm. in diameter. The sap was extracted from those between 3 and 7 mm. in diameter. They were separated from the colonies within 1 day after collection, by breaking up the *Lithothamnion* crust into bits which bore one or more cells (each attached by the characteristic solitary pedicel). These were drained on a towel, and carefully dried with filter paper before the extraction of sap. The tendency of the cells to collapse when punctured (which has been noted before)[3] is such that much of the sap is lost by spurting if the attempt is made to collect it in a capillary pipette. Instead of this, the cells were inverted and punctured at the rounded apex by a glass needle, along which the sap flowed to a bend, where it dropped off into a Pyrex vessel.

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[1] Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1924-25, **7**, 633.

[2] These were erroneously called "*Valonia ventricosa.*" For the analysis of sap from correctly identified *V. ventricosa* see Cooper, W. C., Jr., and Blinks, L. R., *Science*, 1928, **68**, 164.

[3] Blinks, L. R., *J. Gen. Physiol.*, 1929-30, **13**, 223.

Only the sap which flowed out freely was used, and to prevent the expulsion of protoplasm complete collapse was avoided. Most of the cells recovered their original volume and turgor within 2 or 3 days after their return to sea water, showing that the protoplasm had not been seriously damaged.

Over 300 cells from three different collections were punctured, to obtain 25 cc. of sap, which was clear and practically free of chloroplasts. Its specific gravity at 21°C. was 1.0258 as compared with 1.0277 for sea water. These values agree very well with those which Dorcas obtained with sap from the stranded cells. They indicate why the larger cells are able to float when detached from the substrate. (Smaller cells may be held down by the weight of wall, protoplasm, and pedicel; only attached, non-floating cells were used for the extraction of the sap here analyzed. All cells, however, stand upright in sea water, and tug at their attachment like captive balloons.)

II

The details of analysis follow.

a) Sodium and Potassium.—A 10-cc. sample was transferred from the total volume of sap to a 25 cc. volumetric flask, and treated first with 10 cc. of absolute alcohol plus ammonium oxalate in the approximate ratio of 9:1. Then 5 cc. of the Schaffgottsche[4] ammonium carbonate reagent was added and the total volume made up to exactly 25 cc. by adding a drop or two of water (to compensate the contraction due to dilution of the alcohol). The flask was then tightly stoppered to prevent loss of alcohol by evaporation, and set aside long enough to permit all the calcium and magnesium to be precipitated as oxalate or carbonate. The precipitate was removed by centrifugalizing at about 2500 revolutions per minute in covered tubes. Two 10-cc. samples, each equivalent to 4 cc. of original sap, were then withdrawn and the sodium and potassium determined.

After evaporation and gentle ignition in quartz the total weight of salt present was determined. This was assumed to consist of potassium and sodium chlorides exclusively, since only negligible amounts of other anions were found. A test with barium chloride on an acidified 0.5-cc. sample of the sap showed the presence of an unweighable trace of sulfate. Carbonate ion, which might have been present in the original sap, could not have survived the ignition in the ammoniacal solution. Bromide and iodide were found to be absent by treating portions of the sap with chlorine water and extracting with chloroform. Nitrate was found to be absent by the brown ring test.

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[4] Treadwell, F. P., and Hall, W. T., Analytical chemistry, 1924, 6th ed., 2, p. 79.

Potassium was determined as potassium perchlorate. Owing to the small amount present, the weight of the salt was only 0.0035 gm. For this reason the error in this determination may be rather large, ±3 per cent. Since the sodium was determined by difference the value assigned to it is subject to an error due to the error of the potassium in addition to the ordinary error of weighing. However, the amount of sodium is so much greater than the amount of potassium that this extra error is not significant. The sodium value is correct to about ±0.4 per cent.

b) Calcium and Magnesium.—These were determined in a single separate 10-cc. portion of the original sap. The calcium was precipitated as oxalate according to the procedure of Treadwell[5] and weighed as calcium oxide after ignition in platinum. The estimated error is ±1 per cent.

The magnesium was precipitated as magnesium ammonium phosphate from the filtrate of the calcium determination. It was then determined as magnesium pyrophosphate by the method of B. Schmitz.[6] The estimated error is ±0.5 per cent.

c) Halide.—The halide was determined in two samples of 1 cc. each by Mohr titration with silver nitrate. The error of this determination is about ±0.5 per cent.

d) Ammonia and Hydrogen Ions.—Since the samples for the above determinations had been kept in the ice box for 10 days after extraction, a fresh sample of about 4 cc. was extracted for the determination of ammonia, and hydrogen ion concentration. Ammonia was determined in two samples of 1 cc. each, by treating with sodium hydroxide and distilling over into standard acid. One of the standard acid samples was titrated with a standard alkali, and the second was Nesslerized. The amount of acid neutralized by the ammonia was no greater than the possible error in reading the burette. The Nessler test showed a very faint trace of ammonia.

The pH of the fresh sap was found to be 6.2, by use of chlor-phenol red (allowing for salt error).

The transfer pipettes used in this work were all certified by the Bureau of Standards to be correct within the permitted tolerance. The burette used for halide had received a certificate from its manufacturer, showing that it had only negligible corrections. All other volumetric apparatus had been calibrated in this laboratory.

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[5] Treadwell, F. P., and Hall, W. T., *loc. cit.*, p. 86.

[6] Treadwell, F. P., and Hall, W. T., *loc. cit.*, p. 78.

III

The analysis follows:

Chloride	0.6028 moles per liter
Sodium	0.5570 " " "
Potassium	0.0064 " " "
Calcium	0.0080 " " "
Magnesium	0.0167 " " "
Sulfate	trace
Total cations	0.5881 " " " 0.603 gm. equivalents per liter

The molar ratio of potassium to sodium is 0.0114. This is even lower than the ratio found in the stranded cells,[1] which was 0.0278. The exact ratio is apparently variable since determinations made in 1929 gave 0.0314 for stranded cells, and 0.0052 for growing cells. An earlier ratio found by Dorcas in a small sample of sap from growing cells was 0.04. In most of these cases the K:Na ratio differs from that in the sea water, and is as often reduced as it is increased. The best values for the growing cells show a partial exclusion of potassium, its concentration being 0.0029 molar and 0.0064 molar, while the sea water has about 0.0125 molar. Thus the sap has only 0.23 to 0.51 as much potassium as the sea water. The stranded cells accumulate potassium slightly, having 1.3 to 1.4 times as much as the sea water. The new analyses emphasize more than ever the striking deficiency of potassium in the sap of the Bermuda *Halicystis*. In every case its concentration is less than 4 per cent of that in the sap of *Valonia macrophysa*, in which the lowest K:Na ratio found at Bermuda is 2.55 (unpublished data). The ratio runs up to 5.72 in this species, and to 18.5 in *V. ventricosa*.[2] Large differences between the saps of *Chara ceratophylla* and *Nitella clavata* have recently been demonstrated by Collander's detailed analysis[7] of the former, and *Nitella* sap has also been shown to vary considerably in its K:Na ratio.[1,8] In view of these variations it is perhaps not surprising that the sap of the Pacific Coast *Halicystis ovalis* is very different from that of the Bermuda form; its K:Na ratio of 1.5 approaches that of *Valonia macrophysa*.[9]

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[7] Collander, R., *Acta Botanica Fennica*, 1930, 6, 1.

[8] Hoagland, D. R., and Davis, A. R., *J. Gen. Physiol.*, 1922–23, 5, 629.

[9] Brooks, S. C., *Proc. Soc. Exp. Biol. and Med.*, 1930, 27, 409.

In the present state of our knowledge these differences can only be called "specific." The Bermuda form differs markedly from the *H. ovalis* of Europe in morphological details, and reaches a greater size. It is being proposed elsewhere as a new species. In the absence of sap analyses for *H. ovalis* and *H. parvula* of Europe it has a unique position on account of its extreme divergence from the *Valonia* types. Its value in comparative studies is thereby enhanced.

SUMMARY

Analysis was made of 25 cc. of vacuolar sap, obtained from over 300 cells of *Halicystis* collected in their position of growth in Bermuda. The determinations agree very well with those of the stranded cells, except that there is even less potassium present. The K:Na ratio is 0.0114.

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THE VARIATION OF ELECTRICAL RESISTANCE WITH APPLIED POTENTIAL

I. INTACT VALONIA VENTRICOSA

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Many living systems adhere to Ohm's law unless the applied voltage exceeds a certain limit above which the apparent resistance to direct current does not remain constant but decreases (Strohl[1]). This is one of the many resemblances to electrode phenomena which have been pointed out by Gildemeister.[2] Probably all living tissues have a lowered resistance as one of the aspects of injury by electrocution.[3]

An apparent *increase* of resistance, as measured in the Wheatstone bridge during the application of increased direct potentials, has not often been observed, but occurs in several of the large plant cells studied in this laboratory. For example, the resistance of *Nitella*, ordinarily very high in most solutions and constant under 5 to 100 mv. applied potential (unless stimulation occurs[4]) may be lowered by contact with KCl; after this the application of relatively high voltages increases the resistance at the point where the current passes from the external solution into the protoplasm. *Valonia macrophysa*, measured very soon after its collection in Bermuda, may also have a resistance which varies with the applied potential, and is then usually greater when the current passes in one direction than in the other. This seems to be due to injury at one end, where the attachment to a neighboring cell has been broken. *V. macrophysa*, of Tortugas, seldom entirely recovers from the injuries of separation and cleaning and its resistance remains low and variable during its'life in the laboratory (which, in the summer, is usually cut short by zoosporulation).

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[1] Strohl, A., La conductibilité electrique du corps humain, Paris, 1925.

[2] Gildemeister, M., *Handb. norm. u. path. Physiol.*, 1928, **8**, pt. 2, 657.

[3] Injury and recovery after the application of high voltages to plant tissue have recently been studied by Dixon and Bennet-Clark, who also found a small *rise* of resistance to follow after very short stimuli of certain voltages (low frequency alternating current). *Cf.* Dixon, H. H., and Bennet-Clark, T. A., *Proc. Roy. Dublin Soc.*, 1927, **18**, 351; 1928, **19**, 27.

[4] Blinks, L. R., *J. Gen. Physiol.*, 1929-30, **13**, 495.

The variation of resistance with potential is most characteristically shown in the freshly gathered cells of *Valonia ventricosa*. Preliminary notes[5] have indicated the character of this effect, which is here more completely described. Kept in the laboratory, the cells reach a condition in which their resistance is high and does not change either with time or with variations in voltage.[6] We shall call this the "constant state." But for a long period, while this state is being approached, the resistance is low if low potentials are applied and rises when the potential is increased. The amount of the rise is largest at first and becomes less as the cells approach constancy. Some of the smaller cells never show the variability; others which have nearly lost it may be made to display it again in larger degree simply by repeated measurement. Impaled cells show the effect most strikingly[7] and contribute largely to the proposed explanation. Their behavior will be further discussed in another paper.

The experiments here reported were performed with *intact* cells, measured soon after collection at the Dry Tortugas, Florida, in the months of June, July, and August. The temperature of the air, and of the sea water in which the cells lived, was between 25° and 30°C. The methods and apparatus employed in the study of the variable state are the same as used to measure protoplasmic resistance in the constant state, as previously described.[6]

The electrical circuit was an equal ratio-arm Wheatstone bridge with non-polarizable electrodes. In some cases, measurements were made with the string galvanometer connected directly into the bridge without the vacuum tube detector. This was possible on account of the low resistances of *Valonia* cells. The values were read directly from the decade boxes at balance, or, in the case of resistances changing rapidly during current flow, by photographically recording the moving string image. Calibration was automatically obtained at any given value of resistance and potential, by the known increments of resistance (in 100 or 1000 ohm units) which were made to keep the image upon the photographic paper.

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[5] Blinks, L. R., Carnegie Institution of Washington Year Book No. 26, 1926–27, 217; No. 27, 1927–28, 270. *Am. J. Physiol.*, 1928, **85**, 351.

[6] Blinks, L. R., *J. Gen. Physiol.*, 1929–30, **13**, 361.

[7] Blinks, L. R., Carnegie Institution of Washington Year Book No. 28, 1929, 277.

The applied potential was read directly on a millivoltmeter (a Weston milliammeter adapted by series resistance to register one-half the potential drop through the entire bridge). This gave essentially the potential drop across the cell at balance. It was frequently calibrated against a potentiometer, and was easily read to about 5 per cent.

The electrodes were carefully prepared to avoid appreciable polarization at any of the current densities employed. Large sheets of lead, amalgamated with mercury and coated electrolytically with chloride, were dipped into cups filled with acidified sea water. These cups were connected to a second pair of vessels bearing the cell holders, by large blocks of agar imbibed with sea water. These were frequently renewed to avoid diffusion from the electrode vessels.

The cells were supported in circular holes cut in stiff agar blocks which maintained a slight pressure on the ends of the cells. These blocks were imbibed, and the wells filled, with the desired solution (usually sea water) through which the electric current passed in entering and leaving the cell. Leaks of fluid were prevented by covering the tops of the wells tightly with glass or agar slips, the residual seepage being carried away by filter paper drains laid close to the contacts. Under these conditions the excess moisture drained off the cell and its resistance became stationary in 3 or 4 minutes (aside from inherent variations in the cells).

It should be emphasized that neither dead cells, nor air-filled walls, nor any simple electrolytic conductor, showed the effects to be described. Large currents were sent through these for long periods of time without appreciable change of resistance. The variation of resistance with potential was only found in the living cells. Unless otherwise noted, the resistance values of the latter are those attained in the "steady state" after the "transient effects" are over, and no further change in the current is occurring. They thus include any polarization potentials in addition to true ohmic resistance.

A resistance that apparently increases with potential must have a lowered initial value, and this is characteristic of the freshly gathered cells of *V. ventricosa*. Their resistance is often only a third or a half that attained when the cells have reached the constant state. Since the resistance of the cell wall, as a shunt around the protoplasm, remains nearly the same during this time, the changes in the protoplasm itself are relatively still greater.

As an example: a freshly gathered cell, 2 cm. in diameter, and free of adhering growths, had a resistance, when placed in the apparatus, of 700 ohms, rising slowly to 800 ohms, when measured under an applied potential of 20 mv. The resistance rose to 1300 ohms when the potential was increased to 75 mv., and fell off again at potentials above this. It returned to 750 ohms at 20 mv. A cell of similar dimensions, which had stood quietly in sea water in the laboratory for 4 weeks, had a resistance between 2100 and 2200 ohms; this only increased about 5 per cent over the range of 15 to 100 mv. applied potential.

When the first cell was killed its resistance dropped to 20 ohms at all potentials. Its sap and protoplasm were then removed, the cellulose wall was well washed out with sea water, and inflated with air. This wall had an average resistance of 2450 ohms (at all potentials), when measured in the same position as the cell, and under the conditions detailed in the previous paper.[6] This value makes it possible to calculate what the *protoplasmic* resistance was in life. By the method of calculation previously described[6] it was found to have been only 920 ohms when measured at 20 mv., and to have risen to 2770 ohms at 75 mv. Thus an increase of 200 per cent in the living protoplasmic resistance had occurred under the influence of the increased voltage.

Cells showing a smaller variation in resistance have higher initial values for the protoplasm. The isolated wall of the nearly constant cell used for comparison above, had an average resistance of 2380 ohms. Its living protoplasmic resistance was therefore between 18,000 and 29,000 ohms. This approaches the values found in the constant condition of the cells, in which the resistance of the living protoplasm may be 50,000 ohms or more.[6]

The cells usually tend to rise in resistance after collection, but the increase is not always gradual and there are often variations from day to day, apparently brought about by minor mechanical injury. The extreme sensitivity of the cells to handling at this stage is shown by the following example:

A cell was measured immediately after collection, without cleaning or drying. The resistance rose from 1000 to 1200 ohms during 2 minutes of measurement at 25 mv. Ten minutes later, however, the resistance had fallen to 700 ohms at 25 mv. A brief application of 100 mv. brought it back to 1200 ohms, but it fell to 680 ohms at 25 mv. again. The cell was then removed and wiped very gently on a towel for only 30 seconds. On replacing it, the resistance had fallen to 140 ohms, and in 1 minute more was only 60 ohms. Removal to sea water failed to cause recovery and 1 hour later the dead resistance was 40 ohms. The average resistance of the air-filled wall was 1650 ohms, giving for the live protoplasm a maximum resistance of 4400 ohms, which decreased nearly to zero during the experiment.

The cells at this stage are often injured by continued measurement or long sojourn in the apparatus. Frequently a cell which has stood several days without disturbance in sea water has a very high resistance, when first placed between the agar blocks. One cell reached in 3 minutes a resistance, measured at 25 mv., of 3800 ohms, which was slightly increased by application of 100 mv. (to 4000 ohms). The resistance soon began to drop, however, and in 8 minutes had reached 1250 ohms (at all potentials). At 900 ohms it was removed to sea water for recovery. The next day it appeared well, and gave an initial resistance of 3000 ohms at 25 mv. But the value soon decreased, and in 6 minutes was 2000 ohms with the current in one direction, 1800 ohms in the other. 100 mv. caused a rise to 3300, and to 2900 ohms, respectively, and during a long application the resistance rose to 3700 ohms. When the cell was killed 4 hours later the average resistance of the wall was found to be 4100 ohms. This agrees fairly closely with the values of the first measurement (and less closely with the highest later attained under 100 mv. applications).

When we calculate what the protoplasmic resistance had been in the living state, we find that it was between 52,000 ohms and 166,000 ohms at first, but that it fell to 1150 ohms during the measurement. On the second day it had recovered to 11,000 ohms at 25 mv. potential, and the application of 100 mv. raised it to 38,000 ohms. Very large fluctuations had thus occurred during life.

The values of resistance attained under the application of high potentials are shown in Table I, for a group of variable cells. It is seen that the higher resistances reached are from 50 to 90 per cent of the value for the isolated cell wall, and that the values for low potential are in general about half that for high potential. (The differences, of course, fall off as the cells approach the constant state of resistance.)

The ratio of low to high values suggests that the two levels of resistance correspond to two current paths of nearly equal resistance, both of which conduct at low potentials, but one of which becomes non-conducting at higher potentials. There are two circuits in which this is possible. These are shown in Figs. 1 and 2.

In the case of Fig. 1, we assume that the current does not pass across the protoplasm into the vacuole, but that under low potentials it may pass around the cell through the wall, and also through the protoplasm just beneath the wall. Since the protoplasm is of about the same thickness as the wall, the relation of resistance levels would be readily explained by the assumption that it consisted of 3 layers (W being imbibed with sea water), and that the outer layer X admitted current at low potentials, but became highly resistant at higher values (Y remaining a poor conductor at all times). The cur-

TABLE I

Apparent Direct Current Resistance Levels of Variable Valonia ventricosa

Cell No.	Living cells				Dead cells	
	Low value		High value		Resistance*	
	Potential	Resistance	Potential	Resistance	Dead cell	Isolated wall filled with air
	mv.	*ohms*	*mv.*	*ohms*	*ohms*	*ohms*
4	25	2000	100	4600	55	4500
23	22	1200	100	2000	—	—
25	25	1000	100	2100	—	—
27	20	1500	100	2800	38	4200
30	25	1450	100	2350	—	—
32	25	1000	100	1700	27	2500
34	25	3600	50	4300	53	4700
44	25	1280	100	1570	40	2000
45	25	1600	100	2300	40	2100
51	25	2200	75	3800	50	4000
54	25	1600	75	2100	—	3300
55	25	1400	100	1690	—	1700
59	25	830	100	910	—	1200
60 (1st day)	25	1900	150	2700	—	—
" (2nd day)	25	3600	75	3800	63	4150

* These resistances do not vary with the applied potential.

rent would thus find two nearly equal paths at low potentials but only one (the wall) at higher potentials.

This assignment of the current paths is attractive in that it explains how the cells may have a high resistance and still maintain a cell sap of high potassium content by the impermeability of *Y*. It is also partially favored by the failure to detect an increase of sulfate ion in the vacuolar sap even after current under low potential had been passed through cells for 1 hour or more from sulfate sea water[8] (the

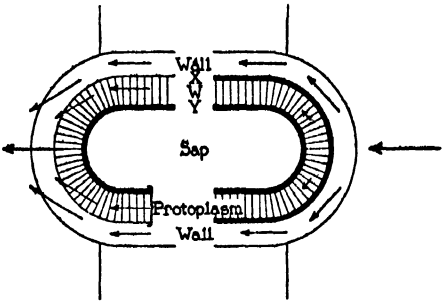

FIG. 1

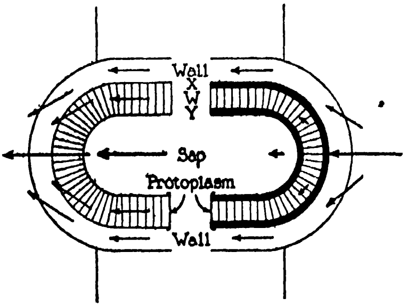

FIG. 2

FIG. 1. Diagram of suggested current paths in the variable cell (the length of the arrows indicates the magnitude of the current). At the left the current is shown equally divided between the wall and the protoplasm. This represents the low resistance value. At higher potentials the condition at the right may be produced, in which the layer *X* has become almost non-conducting so that almost all of the current is confined to the cell wall. In either case, little or no current passes into the sap. (The highly resistant layers are indicated by heavy black lines.)

FIG. 2. Diagram of current paths which represents the apparent condition in variable cells (the length of the arrows indicates the magnitude of the current). At the left much of the current is shown passing across the protoplasm from sap to contact. At higher potentials the condition shown at the right is produced, in which the resistance of the protoplasm has greatly increased, confining most of the current to the cell wall. (The highly resistant layers are indicated by heavy black lines.)

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[8] This had the following composition:

Na_2SO_4	0.4 M	1000 parts
K_2SO_4	0.4 M	20 parts
$MgSO_4$	0.6 M	116 parts
$CaSO_4$	(saturated)	

resistance remaining at the low level throughout). But calculation shows that the migration of any ion would be extremely small over this period at the necessarily small current density employed. The resistance rises in larger currents and a much longer exposure in the apparatus is usually injurious to the cells. On the other hand the less certain criteria of alternating current measurements show that the differences between the low and the high levels of resistance are not paralleled by the expected reduction of capacitance which would be caused by a new layer of dielectric (*X*) added in series to the existing one (*Y*) when the cells passed from the variable to the constant state.

Fig. 2 shows assumed conditions in better agreement with the facts, especially those derived from the study of cells impaled on capillaries. In such cells there is no other path for the current but that directly across the protoplasm, from sap to wall. Yet the same rise of apparent resistance takes place there as in the intact cells. Complete discussion of these results must be deferred, but it may be said that their essential features are duplicated in the experiments performed on intact cells which have been chloroformed at one end. These give virtually a contact with the sap at the killed end, and the current passes across but one layer of living protoplasm.

The tightly fitting contact of the cell with the agar makes it possible to apply chloroform at one end so that it cannot reach the other end except by diffusion through the sap. This is so slow that for a few minutes after the chloroformed end is completely dead the other end is nearly normal. In performing the experiment sea water saturated with chloroform is substituted for the sea water in the well, or a small drop of chloroform may be introduced into the well, which is then covered again.

The prompt effect of the chloroform is to cause a deflection of the bridge detector, corresponding to a potential difference in the cell up to 20 or 25 mv., the killed end being positive. (This is in agreement with the impaled cells of *V. ventricosa*, in which the inside (sap) attains a value of 15 to 25 mv. positive.[7]) This potential difference is balanced out by a series potentiometer, or the deflected state may be used as a pseudo-zero.

When the variable cells are thus chloroformed at one end quite different resistances are found, depending on the direction in which the current flows through the cell. These become most marked at the higher potentials. Below 20 mv. the resistance is somewhat less than in the unchloroformed cell and remains about the same with the current passing in either direction. But as the positive current is increased *outward* across the living end, the resistance begins to *decrease* with increasing potential, and may be nearly halved at 100 mv. Only when the positive current is passed *inward* across the uninjured protoplasm does the resistance *rise* with potential.

The variable and directional effects are most marked for about 10 or 15 minutes after chloroforming and then begin to fall off as the total resistance decreases. This is taken as meaning that the chloroform has begun to injure the other end of the cell, which eventually is also killed. Before this occurs, however, the effects are sufficiently clear to explain the essential phenomena of current flow across a single layer of protoplasm. The results are shown diagrammatically in Fig. 3. *A* is the resistance curve of cells in the constant state. For a range of over 100 mv. either side of zero, the resistance is constant. It falls off as the breakdown value is exceeded, but more rapidly when the positive current passes *outward* (from sap to sea water) than when it passes *inward*. Curve *B* represents the variation of resistance in a cell which has nearly recovered constancy. The maximum rise of resistance between zero and 100 mv. with inward current is only about 5 per cent, but the resistance falls rapidly when the current passes outward. Curve *C* is that of the typical variable cell, in which with inward current the resistance rises over 100 per cent with the increase of potential between zero and 100 mv. An outward current causes a smaller decrease of resistance. Curve *D*, which represents an impaled cell, is included to show the extreme of the variable condition, in which the resistance is nearly at zero with small currents in either direction, but increases greatly with larger inward currents. Increased outward currents, on the contrary, have very little effect, since the resistance is already very low. Curve *E* shows that in dead cells the variations are absent, and the resistance is low at all potentials.

The shape of these curves is rather variable, and depends in large degree on the immediately preceding history of the cell. If a current has been passed shortly before, the whole curve may be shifted to the right or left within the limits represented by *B* and *D*, depending on the direction in which the current has passed (an explanation of this will be suggested presently).

All the levels mentioned are those of the steady state in which the resistance has reached a value that no longer changes with time during the current flow. But the speed with which this steady state is attained is highly dependent on the previous treatment, and varies with the length of time between applications of potential. Nothing has previously been said of the time course of these resistance changes, and they are more properly reserved for discussion in another paper with the "transient effects" which occur at every application and removal of potential across the cells. They are, however, of value in analyzing the cause of the resistance rise.[9]

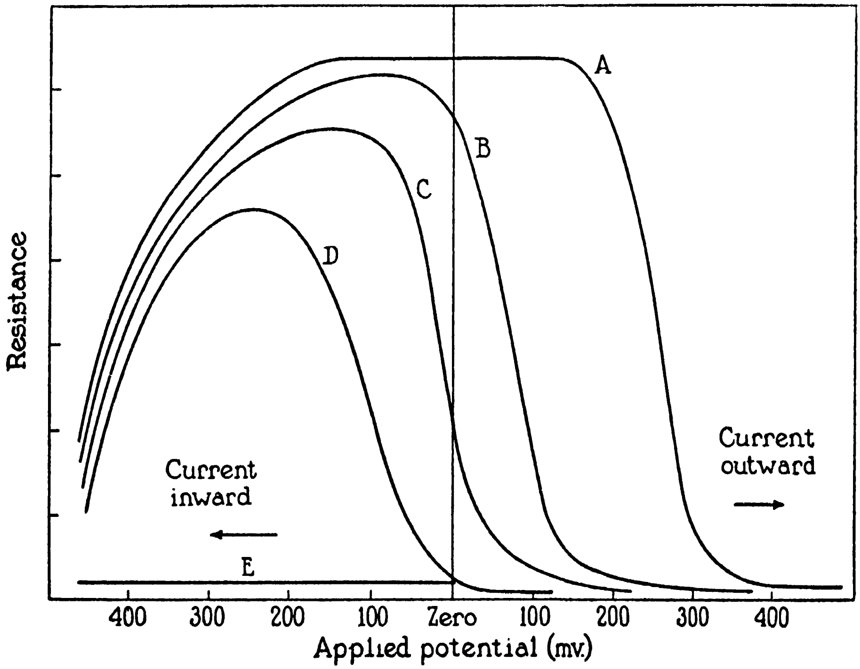

FIG. 3. Diagrammatic graph showing the variation of resistance with potential across a single layer of *Valonia* protoplasm. The resistance is in arbitrary units, the applied potential is in millivolts, increasing on either side of zero; to the left there is an increasing positive current from sea water to sap; to the right, from sap to sea water. Curve *A* represents the constant condition, *B* and *C* the variable condition of intact cells, *D* that of an impaled cell, and *E* that of a dead cell.

[9] Blinks, L. R., *Proc. Soc. Exp. Biol. and Med.*, 1928–29, **26**, 359. An expansion of this preliminary note is shortly to be published.

A very small current may pass through the cells for a long time—up to an hour or more at 20 mv.—without effect either on the resistance or the back E.M.F. A slight increase of potential above this is also ineffective at first. But as the current flow continues the galvanometer deflects, at first slowly, then more rapidly, and finally more slowly again, to reach what resembles a steady state. If the measuring potential is now removed, a much larger back E.M.F. is discovered,

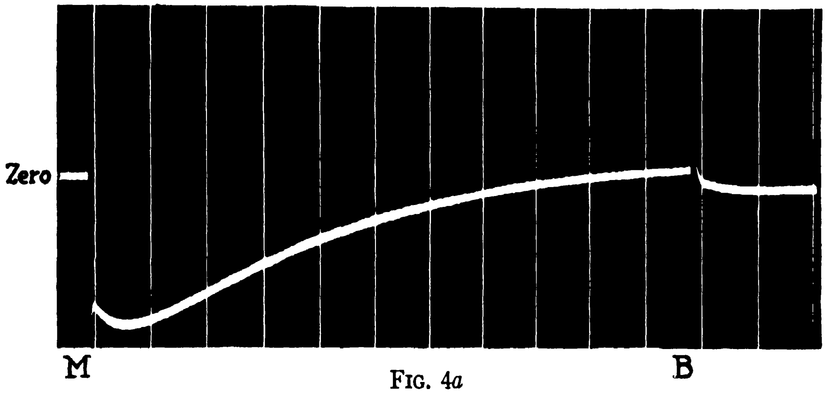

FIG. 4*a*

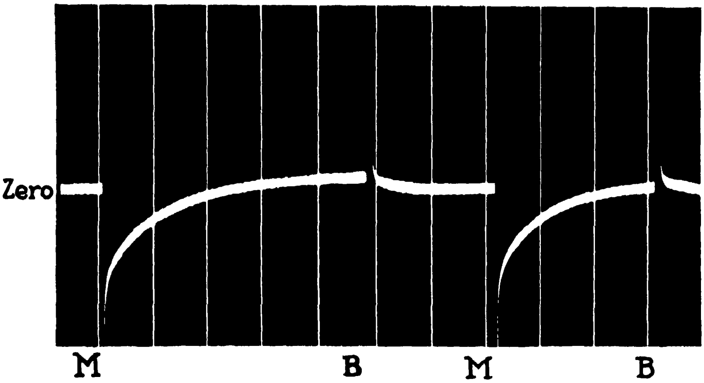

FIG. 4*b*

FIG. 4*a*. String galvanometer record of the resistance rise under the application of 75 mv. to a variable cell. The bridge has previously been balanced to the steady state value so that the curve rises to zero. *M*, make, *B*, break, of voltage applied to bridge. Time marks, 1 second.

FIG. 4*b*. (Continuation of 4*a*.) String galvanometer record of second and third applications of 75 mv. in immediate succession after 4*a*. Details as in 4*a*.

and a current flows for a moment in the opposite direction, the zero line being approached by a regular curve.

The application of the same potential again causes a regular rise of apparent resistance, but this time more rapidly, showing that an effect of the previous exposure persisted. A third application in quick succession is usually still faster. Fig. 4 shows a typical string galvanometer record of such a series of applications.

Several facts come out of the study of such records. It is evident that part or all of the apparent rise of resistance is due to a back E.M.F. developed by the current. This has the effect of a capacity (either polarization or static) but it differs from these in that the time constant is quite different for the charge and the discharge, the first charge taking 10 to 20 times as long as the discharge. The time constant of charging also changes from one exposure to the next, becoming less with succeeding applications. Even after several exposures have been made, the curves do not reach that equality and symmetry of rise and fall which characterize condenser and electrode charge and discharge (the "superposition law" of Curie[10]). Since cells in the constant condition have very symmetrical charge and discharge curves (*cf.* the curves for *Nitella*[4]), it seems probable that another process is occurring in the first part of the resistance rise in the variable cells. This in effect is a change of the protoplasm from an electrode fairly reversible to the ions passing, to an electrode nearly or entirely irreversible to them, and finally becoming polarized with an opposite and nearly equal E.M.F. The question occurs whether it is the nature of the protoplasmic electrode that is being changed (as if by a decrease in pore size) or whether the kinds of ions in the current are changing. The latter is the simpler explanation, and has considerable evidence in its favor. The mechanism for this effect is suggested by the thin collodion membranes discussed in another paper.[9] In these the resistance is found to be dependent on the applied potential, rising when sodium ions are carried across it, and falling when potassium ions are so carried. In such collodion the resistance rise is real, due to the different ion mobilities, and not to a back E.M.F. of polarization. But the mechanism is suggestive of what might happen in a membrane which allowed one kind of ion to pass freely, while polarizing when presented with another kind.[11] Small currents would not carry away the first kind any faster than they diffused back, but greater currents would decrease their concentration, and polarization would then occur. In so-called "non-polarizable" electrodes an apparent increase of resistance may be brought about by large current densities in this manner.

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[10] Curie, J., *Ann. chim. et phys.*, 1889 (6), **18**, 203.

The question remains, what are the ions which the protoplasm allows to pass in its variable state? The high concentration of KCl in the sap, and the fact that the resistance falls and polarization decreases, when the current passes outward from the sap across the protoplasm suggests that it is potassium ions which have a high mobility in the variable *Valonia* cells. There is considerable evidence that potassium remains trapped for a long time in the protoplasm, and its presence is apparently responsible for the shift of the curves in Fig. 3 toward *D*. As the cells recover, this potassium is lost by diffusion or by absorption into the vacuole, and the highly resistant level of *A* is reached. However, application of 0.6 M KCl to the outside of the cells produces very little effect for considerable time, and there is even the anomaly, during part of the exposure, of a shorter time constant of polarization in KCl than in sea water. But it should be said that great difficulty is met in interpreting the effects of single salts, since they produce alterations in the protoplasm. A large amount of KCl in sea water sometimes has an effect in preventing the rise of resistance in the variable state, but this disappears as the cells approach the constant state. Further evidence on the reversibility to ions will be given for impaled cells in another paper.

The ultimate cause of the variable state has been ascribed to mechanical injury,[6] but the other alternative might be to regard the variable state as the natural, growing condition of the cells, as opposed to an artificial, highly impermeable state which they may reach in the laboratory. However, many cells have a constant high resistance from the beginning, and can later be made variable by mechanical injury such as impalement.[7] Reference has been made in a preceding paper[6] to the ease with which *V. ventricosa* protoplasm breaks up into

[11] *Cf.* Labes, R., *Arch. exp. Path. u. Pharm.*, 1927, **125**, 29; Zain, H., *Ibid.* 53; Labes, R., and Zain, H., *Ibid.*, **126**, 284, 352.

hundreds of tiny spheres which are new cells inside the old wall. It is easy to conceive this process as partly occurring, the strains in the surface greatly changing its permeability (or thickness) without gross rupture. High temperature alone (up to 30° or 31°C. at Tortugas) does not cause the variable state, since constant cells taken back from New York to Tortugas and exposed to this temperature do not develop the effect, and the recovery of variable cells takes place regularly at this temperature.

Since the variable condition is a means of producing great changes of permeability to some ions, during an apparently healthy state of the cells, it has a bearing upon injury and recovery. This will be discussed in a later paper.

Grateful acknowledgment is made to the Carnegie Institution of Washington for its generous cooperation at the Tortugas Laboratory, where these experiments were carried out.

SUMMARY

Many of the freshly gathered cells of *Valonia ventricosa* have a resistance to direct current which is variable and depends on the potential applied. It is low when low potentials are applied and rises sharply at higher values. The rise may be more than 100 per cent in the cell as a whole, which is equivalent to several hundred per cent in the protoplasm alone. The rise becomes less as the cells stand in the laboratory, until a maximum is reached at all applied potentials, low and high, below the breakdown value (about 100 mv.): the cells are then said to be in a constant state.

During the variable state, the resistance rises when the positive current enters the protoplasm from outside, and falls when it passes out from the vacuole (this is determined by killing one end with chloroform).

The rise of resistance becomes faster with closely succeeding applications of potential. This is ascribed to the removal from the protoplasm of ions to which it is reversible. There is some evidence that these may be potassium ions.

Much of the apparent resistance rise may be accounted for by a back E.M.F. caused by the flow of current.

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A SCREENED BRIDGE FOR THE MEASUREMENT OF ELECTROLYTIC CONDUCTANCE. I. THEORY OF CAPACITY ERRORS. II. DESCRIPTION OF THE BRIDGE

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INTRODUCTION

Kohlrausch's classical method for measuring the electrical conductance of solutions has had the benefit of numerous improvements by many workers. The researches of Washburn[1] were particularly valuable in stimulating accurate conductance work in this country. Morgan and Lammert[2] have discussed some of the sources of error in the electrical arrangement used in the method, with valuable references to previous work. Of particular importance from the standpoint of precision measurements has been the comparatively recent introduction of vacuum tube alternating current generators and amplifiers for providing currents of symmetrical wave form, on the one hand, and increasing the sensitivity of the detector on the other.[3] Jones and Josephs[4] have described in a very detailed paper a direct reading alternating current bridge embodying these new features. Their paper also includes a valuable and comprehensive study of various sources of error from the electrical standpoint in the Kohlrausch method as it has been generally used.

Important features of Jones and Josephs' bridge are the abandoning of the circular slide wire (which is capable of introducing a disturbing unsymmetrical inductance in the measuring circuit) and the use of a suitable earthing device for the bridge for minimizing disturbing earth capacity effects. They have, however, condemned the use of electrostatic screening of the bridge.[5] The use of screening, when properly designed, for the entire bridge has decided advantages. Since the readings are unaffected by the movements of the observer, a screened bridge is more convenient to operate than an unscreened one. Without adequate shielding, delicate balances are difficult to make due to shifting capacities introduced by the hands of the observer, etc. Also, with suitable screening, measurements of high precision are possible under conditions existing in modern laboratories in which other electrical work is certain to be in progress. Under such conditions the use of an unshielded bridge is at times troublesome if not impossible.

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[1] Washburn and Bell, THIS JOURNAL, 35, 177 (1913); Washburn, *ibid.*, 38, 2431 (1916); Washburn and Parker, *ibid.*, 39, 235 (1917).

[2] Morgan and Lammert, *ibid.*, 48, 1220 (1926).

[3] Hall and Adams, *ibid.*, 41, 1523 (1919); Jones and Bollinger, *ibid.*, 51, 2407 (1929)

[4] Jones and Josephs, *ibid.*, 50, 1049 (1928).

In Part I of this paper the theory of disturbing capacity effects in an alternating current Wheatstone bridge circuit is discussed, with reference to the underlying principle of the apparatus as a measuring instrument. Also, the basis for designing proper screening is developed.

In Part II, a shielded alternating current bridge for measuring electrolytic conductance with a high degree of precision is described. The main problem in the design of such a bridge is the fixing and the proper balancing of "stray couplings" in the apparatus.

I. The Theory of Earth Capacity Errors in the A. C. Wheatstone Bridge

1. Stray Couplings.—When a direct current flows through a conductor, leakage can take place only through inadequate insulation. With alternating currents, the case is more complicated because the current may also leak away through electrostatic and electromagnetic linkings, commonly known as "stray couplings" with the surroundings. Such stray couplings may be electrostatic capacities between the parts of the circuit and capacities between these parts and the ground. There may also be mutual inductive effects due to linkages with stray magnetic fields. The effects produced by these couplings vary with the frequency, being more troublesome at higher frequencies.

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[5] Their conclusions are based on tests made with a screen placed near one branch of the bridge network, which introduced an *unbalanced* earth capacity effect into the circuit. Such a test does not prove, however, that electrostatic screening cannot be used without error if it is properly designed.

Stray electromagnetic couplings are particularly important in the case of parts of circuits, such as transformer coils, which have appreciable inductance. These mutual inductive couplings may be reduced by using materials of high permeability in the cores of the transformers, by shielding stray fields with screens of high permeability, or by sufficiently separating the branches of the circuit having high inductances from each other. In the case of a Wheatstone bridge for measuring electrolytic conductance, the only branches in the network capable of very serious inductive coupling with each other are the detector and the oscillator. The best plan is to avoid the necessity of electromagnetic screening, which is always difficult and seldom completely satisfactory. This is readily accomplished by removing the oscillator to a sufficient distance from the detector, which is more conveniently located near the bridge, and avoiding as much as possible the introduction of stray inductance in the bridge itself. A distance of 10 to 15 feet between the detector and the oscillator is usually sufficient.

Electrostatic couplings existing between various branches of a circuit, directly or to ground, cannot be eliminated entirely in this simple manner without introducing another difficulty. The difficulty is that excessive separation of all the branches of the bridge from each other and from ground, besides being inconvenient, introduces additional inductance into the network because of the increased length of leads required. The result of this increased looping is that although capacitative couplings are reduced, inductances capable of disturbing the bridge balance are thus added, since the bridge balance may be changed by the inductive effect of varying stray fields enclosed.

In Fig. 1 is shown a diagram of a bridge supplied with alternating current, indicating the position of some of the capacitative couplings. The capacity paths from one lead of the oscillator to the other, either directly (C_1) or through ground (C_a and C_c in series), merely shunt the current input, and do not affect the bridge balance. Similarly, the coupling between the leads to the detector does not influence the bridge reading, merely shunting the detector. However, couplings are possible which may disturb the balance of the bridge. For example, current leaking from the branch AD, through CB to the terminal B, and back to the oscillator through the branch BC, would have an effect on the bridge balance. (It should be pointed out that the bridge terminals A, B, C and D include the leads appended to them since if they are not too long their resistance is usually negligible.) Although some of these numerous possible paths will tend to neutralize each other, the effects of these paths must be controlled; otherwise, the bridge readings will depend on the relative positions of the units in the circuit and on the surroundings.

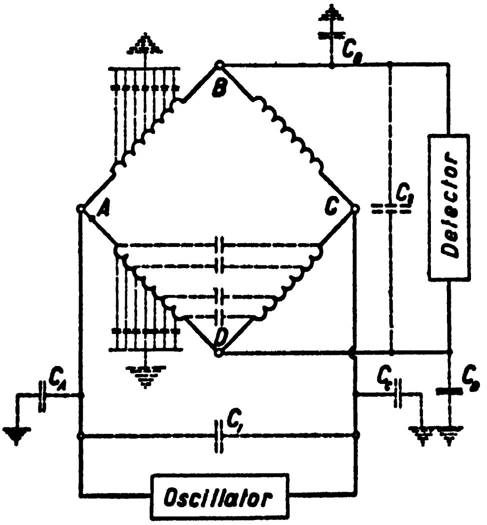

FIG. 1

Precise measurements are not possible unless these troublesome couplings are neutralized by being made symmetrical with respect to the terminals of the detector. It is not possible to avoid electrical asymmetry entirely in the construction of the apparatus, particularly if the bridge is used for measurements with various frequencies of current, requiring readjustments in the oscillator, or perhaps different oscillators. Neither is it practicable to reduce these couplings so as to make them negligible, especially at the higher frequencies, and in the measurement of dilute solutions having large resistances. For these reasons it is necessary to provide means for controlling and balancing these disturbing effects. This is accomplished (a) by properly designed shielding[6] and (b) by the "Wagner earthing device," both of which will be discussed below. Shielding makes it possible to fix definitely and to control the possible interbranch capacities. Before discussing the effect of capacity couplings on the bridge as a whole, it is first necessary to consider the effect on the impedance of a single branch due to the capacity between it and the ground (or a surrounding screen).

2. *Effect of Earth Capacity on a Conductor.*—In general, with a conductor carrying alternating current, there will be present a distributed capacity of total magnitude c to earth, or to a surrounding screen, as indicated in Fig. 2a, where OR is the conductor of impedance[7] R, and S is the ground or screen. In Fig. 2b is shown a special condition which will help in the consideration of a more general case. In this special case the total capacity from OR to S is concen-

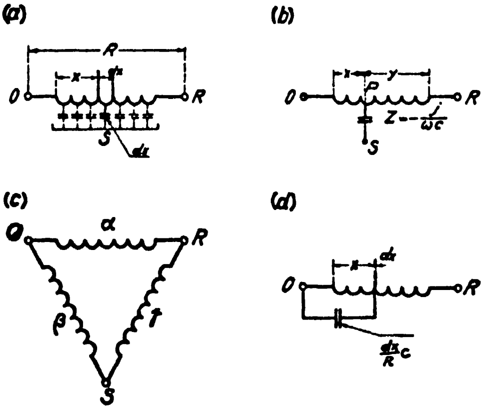

FIG. 2

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[6] The necessity of electrostatic screening for measurements at frequencies of, say, above 10.000 cycles is generally recognized. See for example G. A. Campbell, "The Shielded Balance," *Electrical World*, April, 647 (1904); W. S. Shackelton, "A Shielded Bridge for Inductive Impedance Measurements," *Bell System Technical Journal*, **6**, 142 (1927); J. G. Ferguson, "Shielding in High Frequency Measurements," *ibid.*, **8**, 560 (1929).

[7] Ohm's law can be applied to sinusoidal alternating currents if vector symbols instead of scalar symbols are used. On this basis the impedance operator, which is analogous to resistance in D. C., is defined as the vector voltage divided by the vector current. See B. Hague, "A. C. Bridge Methods," pp. 32–35; R. M. Wilmotte, *Phil. Mag.*, **6**, 788 (1928).

trated at some point P, between O and R. Let [x] be the impedance from O to P, [y] the impedance from P to R and [z] the impedance from P to S. Let us now substitute for the arrangement connecting the points O, R and S as in Fig. 2b, the arrangement shown in Fig. 2c which we shall prove can be made equivalent by the "Kennelly $\tau - \pi$ transformation."[8] In Fig. 2c [α], [β] and [γ] are the impedances connecting the three terminals of interest, O, R and S. By equivalence of the two circuits (shown in Figs. 2b and 2c) is meant that the potentials of O, R and S are the same at any instant in both arrangements. Thus, for example, the impedance operators $x + y$ must be equal to α and $\beta + \gamma$ in parallel. (The letters in brackets [x], [α], etc., refer to impedances; x, α, etc., refer to the corresponding impedance operators.) We consequently get the following relations

$$\frac{1}{x+y} = \frac{1}{\alpha} + \frac{1}{\beta+\gamma} \tag{1}$$

$$\frac{1}{x+z} = \frac{1}{\beta} + \frac{1}{\alpha+\gamma} \tag{2}$$

$$\frac{1}{y+z} = \frac{1}{\gamma} + \frac{1}{\alpha+\beta} \tag{3}$$

Solving, we obtain the equations

$$\alpha = x + y + \frac{xy}{z} \tag{4}$$

$$\beta = x + z + \frac{xz}{y} \tag{5}$$

$$\gamma = y + z + \frac{yz}{x} \tag{6}$$

(*a*) *Capacity Concentrated at the Midpoint.*—Now, referring again to Fig. 2b, if we consider R a resistance with its total capacity to the ground (S) concentrated at the midpoint, $x = y = R/2$; $z = -j/\omega c$, where c is the total capacity to ground. $\omega/2\pi$ is the frequency and j is the "reactance" operator.[9] Substituting these values in (4), (5) and (6) we obtain

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[8] This transformation has been used by Butterworth [*Proc. Phys. Soc.*, **33**, 312 (1921); *ibid.*, **34**, 8 (1922)] and more recently by Bartlett [*J. Sci. Instruments*, **6**, 277 (1929)] in dealing with the effects of earth capacities.

$$\alpha = R + j\,\frac{R^2\omega c}{4} \tag{7}$$

$$\beta = \gamma = \frac{R}{2} - j\left(\frac{1}{\omega c/2}\right) \tag{8}$$

That is, due to earth capacity effects, the resistance R between the points O and R is modified by a reactance $R^2\omega c/4$ (this may be considered to be an inductance of magnitude $R^2c/4$ in series with the resistance R) and by the impedances (8) from O and R to ground (S) (these may be considered to be capacities of magnitude $c/2$ in series with a resistance $R/2$).

(*b*) *Capacity Uniformly Distributed.*—The more general case of a distributed capacity, as shown in Fig. 2a, is somewhat more complicated to deal with rigorously. However, the vales of α, β and γ for this case are approximately represented by Equations 7 and 8. This assumes, of course, the approximation that the total distributed capacity is represented by a single capacity from the middle of the arm to the screen. If the capacity is uniformly distributed, the effect on α due to the differential capacities $(\mathrm{d}x/R)$ (c) from the differential proportions $\mathrm{d}x/R$ (see Fig. 2) is obtained from Equation 4 to a first approximation by integration as follows[10]

$$\mathrm{d}\alpha = x + R - x + j\int \frac{x(R - x)\omega c\,\mathrm{d}x}{R} \tag{9}$$

$$\alpha = R + \int_0^R \frac{x(R - x)\omega c\,\mathrm{d}x}{R} = R + j\,\frac{\omega R^2 c}{6} \tag{10}$$

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[9] $(j)^2 = -1$. The reactance due to a capacity c is $-(j/\omega c)$; the reactance due to an inductance L is $j\omega L$. See, for example, B. Hague, "Alternating Current Bridge Methods," p. 35.

[10] For a more rigorous treatment of this uniformly distributed case, see A. E. Kennelly, "Electric Lines and Nets," McGraw-Hill Book Co., New York, pp. 61, 395. The method of integration (Equations 9 and 10) used below for obtaining α in this distributed case amounts to neglecting higher order terms in the rigorous treatment which involves the use of hyperbolic functions. However, no such approximations are involved in obtaining Equations 7 and 8.

Equation 10 shows that the resistance of OR, due to the distributed earth capacity effect, behaves as a resistance R in series with an inductance $R^2c/6$.

(*c*) *Impedance Directly Connected to Earth or Screen.*—Another case of interest is one in which the ground or screen S is connected to one terminal of the impedance (Fig. 2d), since screens are often so connected, and bridges are frequently grounded directly from one terminal. The effect due to the capacity from a differential section to the screen S (or earth) is a shunting impedance [dz]. As before, $-j(R/\omega c\mathrm{d}x) = \mathrm{d}z$.

The total impedance operator, then, from O to R is

$$Z_{(\mathrm{OR})} = \frac{1}{\frac{1}{x} + j\,\frac{\omega c\mathrm{d}x}{R}} + R - x = x - j\,\omega cx^2\mathrm{d}x + R - X = R - j\,\frac{\omega cx^2\mathrm{d}x}{R} \qquad (11)$$

neglecting terms of higher order.[11] Integrating from $x = \mathrm{O}$ to $x = \mathrm{R}$

$$Z_{\mathrm{OR}} = R - j\,\frac{\omega cr^2}{3} \qquad (12)$$

In this case we see that the earth capacity effect introduces a negative inductance in series with it of magnitude $R^2c/3$.

3. Earth Capacity Effect on the Bridge Circuit.—In the light of the discussion in the previous section we may now view the effect of "stray couplings" on the bridge network. We shall assume that *direct* couplings between the branches have been avoided by shielding. Assuming, then, that the branches of the bridge are so shielded from each other by screens, and making the Kennelly $\tau - \pi$ transformation for every branch, the bridge with its capacity couplings to ground can be represented as in Fig. 3. The segments L_1, L_2, etc., represent the inductive effects discussed above produced in the branches by the earth capacities. AE, BE, CE and DE and the impedances from the four bridge terminals to ground at E. Each of these impedances includes the separate earth capacity effects from the three branches

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[11] This derivation involves the approximation that the differential capacity effects act independently. For a more rigorous derivation see R. Davis, *J. Sci. Instruments*, 5, 306 (1928).

(corresponding to the various β and γ terms, Equation 8) meeting at each terminal of the bridge network. In addition, there are the capacities to earth from the terminals (and leads) A, B, C and D. For instance, (1) is a resistance in series with a capacity due to AB; (2) is a similar, though not necessarily equal, impedance due to AD; (3) is another one due to the oscillator branch; and (4) is a capacity from A to E, due largely to the lead from the oscillator. All these in parallel constitute AE.

It should be noted that the representation of the bridge in Fig. 3 takes into account the distributed dissipations of current from the branches in the circuit to the screens and earth. The advantages of the artifice used in this representation are that it shows the effects produced on the branch impedances from the standpoint of the four terminals of the bridge, making clear the necessary conditions for proper bridge balance, and shows the limitations which must be observed in designing suitable screening so as not to introduce errors in the measurements.

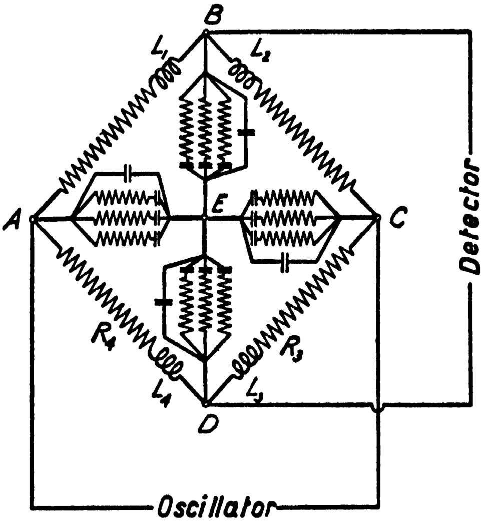

FIG. 3

When the bridge is balanced, the terminals B and D are at the same potential, both in magnitude and in phase. That is, the potentials are equal at every instant and no current flows between the two points through the detector. Referring to Fig. 3, if V is the potential between two points at balance

$$V_{AB} = V_{AD} \text{ and } V_{BC} = V_{DC} \tag{a}$$

This gives by the Vector Ohm's Law[12]

$$\frac{I_{AB}Z_{AB}}{I_{BC}Z_{BC}} = \frac{I_{AD}Z_{AD}}{I_{DH}Z_{DC}} \tag{b}$$

where I is the current and Z the impedance. Now, if the points B and D are brought to the same potential as the ground E, there will be no current flowing in either BE or DE, and the conditions

$$I_{AB} = I_{BC} \text{ and } I_{AD} = I_{DC} \tag{c}$$

will be fulfilled. (There are only three possible paths for current from B or D, when no current flows in the detector.)

Then

$$\frac{Z_{AB}}{Z_{BC}} = \frac{Z_{AD}}{Z_{DC}} \tag{d}$$

If no errors are to be introduced into the measurements, the ratio arms (AD and CD) should be identical in all respects in the case of a direct reading (equal ratio) bridge ($Z_{AD} = Z_{DC}$). Not only must the resistances R_4 and R_3 be equal, but the inductances L_4 and L_3 must also be equal. The latter need not, however, necessarily be negligible, since it is quite feasible to have two equal coils, of similar construction, similarly screened so that, practically, $R_4 = R_3$ and $L_4 = L_3$, in which case $Z_{AD} = Z_{DC}$. Nevertheless, slight differences, in resistance particularly, are likely to occur. This, as well as slight unbalances in the L terms due to screen capacity, are automatically corrected for if two readings are taken, one with the ratio arms reversed. The average value (strictly speaking, the geometric mean) gives the correct measurement.

However, the characteristics of the measuring branch and of the unknown (cell) cannot be considered identical as in the case of the ratio arms. That is, it is not possible to shield the two with relatively close screens, and feel assured that the capacity effects are similar in both cases. However, shielding is desirable from the standpoint of screening out external influences which may affect the circuit. The correct procedure, therefore, is to shield these branches not closely, but at a sufficient distance to make the effect of the capacity on the impedance negligible under the limiting conditions existing in measurements. If the upper frequency range does not exceed 4000 cycles, direct test showed that a separation of 2.5 inches between the coils of the resistance box (or cell) and the shield introduces a negligible error. That is, the terms L_1 and L_2 in Fig. 3 are negligible.

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[12] See Hague, Ref. 9, p. 32.

If these conditions for the ratio arms and the other two branches in the bridge are fulfilled (L_1 and L_2 negligible, $L_3 = L_4$, $R_4 = R_3$, no current in BE or DE) then Equation d reduces to .

$$Z'_{AB} = Z'_{BC} \tag{e}$$

(Z' is the impedance free from earth or screen effect)[13] which is the correct balance condition for an equal ratio bridge without error. Jones and Josephs[4] state in their paper that the necessary condition for the validity of Equation e is that no current whatever escape from any of the bridge parts. Of course, if that is so, correct balance is obtained at once. Practically this is not possible. However, as shown above, correct balance, without error in the measurements, is quite attainable when the proper precautions are observed in spite of a certain amount of current leakage from at least some of the branches.

4. *The Wagner Earthing Device.*—One of the conditions for the validity of Equation e is that the terminals B, D and E (Fig. 3) be at the same potential. This may be done by adjusting the earth impedances AE and CE until no current flows in the detector connected from E to B (or D), when the main bridge (ABCD) is also balanced. That is, $Z_{AE}/Z_{CE} = Z_{AB}/Z_{CB} = Z_{AD}/Z_{CD}$. This is the principle of the device. Since Z_{AE} and Z_{CE} are mixed impedances, consisting of resistance and capacity, the adjusting impedance must similarly comprise a variable resistance and reactance. The reactance

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[13] This impedance may be a pure resistance. The somewhat more complicated case of a cell for measuring electrolytic conductance will be discussed in a later paper. However, if the cell impedance is of sufficiently low-phase angle, it can be regarded as a resistance.

may be either a capacity or an inductance, or both, since any impedance may be balanced by such combinations. It is merely necessary to adjust the impedances Z_{AE} and Z_{CE} to the proper ratio.

The scheme used by Jones and Josephs,[4] who, however, do not discuss the theoretical basis of the device, is a very convenient one. It consists of shunting AE and CE with resistances permitting of sufficient variation in adjustment, and a variable condenser from A to E, or C to E, as required. The advantage of their method lies principally in the fact that for the same delicacy of adjustment condensers of lower capacity can be used than would be required with series resistance and capacity combinations.[14]

An examination of Fig. 3 shows the limitations of connecting the ground point E directly to B or D, and securing balance by the adjustment of a condenser in parallel with the necessary branch. Evidently, except in the case of accidental balancing between AE and CE, the bridge reading will not be free from error. The magnitude of the error will depend on the magnitudes of AE and CE and on the extent of their unbalance. It is, however, possible to determine the unbalance between AE and CE, and then to calculate the error thus introduced in the measurements. This scheme, though feasible in certain types of impedance measurements, is inconvenient since it requires an estimation of the earth capacity with subsequent calculations. For a bridge designed to measure electrolytic conductance the Wagner device is simpler and preferable.

Part II. A Screened Alternating Current Wheatstone Bridge

The shielded bridge, which was discussed from the theoretical standpoint in Part I, is shown diagrammatically in Fig. 4. The detector and oscillator are connected to the bridge by duplex cables inclosed in flexible copper shielding which is grounded. The oscillator and the earthing circuit W_R, W_C are connected directly to terminals of the measuring branch and of the conductivity cell, at M and N.

1. Balancing Condensers.—A twin (double) variable air condenser K is connected across the measuring resistance (MS) and the cell (BN) as shown, for balancing out the reactance of the cell, due to polarization at the electrodes. The ordinary type of single variable condenser has a disadvantage. It is sometimes impossible to get complete balance in the bridge with it, owing to the fact that the residual zero capacity is too large for the required setting. The twin type, which makes it possible to remove capacity from one side, adding it to the other, avoids this difficulty, and does not require a switch for shifting the condenser from one branch to the other. The entire condenser K consists of two such twin condensers. One is of $250\mu\mu$f. capacity, easily reconstructed from the "tandem type" condenser made by the General Radio Co. The other, connected in parallel with the first, is similarly constructed from two micro condensers. The latter has a maximum capacity of about $10\mu\mu$f. and it is provided with a "Vernier" control knob, allowing of very delicate adjustment.

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[14] Jones and Josephs deserve the credit for describing the proper use of the earthing device for balancing the earth admittances in a conductance bridge for work with solutions, although the underlying principle of the method is due to Wagner.

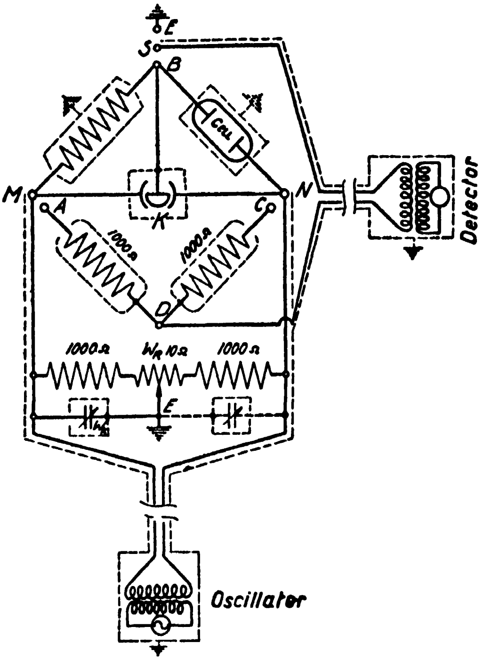

FIG. 4

2. *Radio Arms.*—The ratio arms are connected to the bridge by means of a double pole double throw reversal switch (not shown in Fig. 4). In making determinations the mean of readings with the ratio arms in the two reversed positions is taken. This procedure corrects for slight variations in the resistances (and reactances) of the ratio coils, and avoids the necessity of adjusting them to perfect equality. The ratio coils are surrounded by local symmetrical shields connected to the junction point D. The shield localizes the total distributed capacity of the ratio arms at point D, and avoids the possibility of any stray couplings directly with these important branches of the bridge.

The ratio arms consist of two closely matched Ayrton-Perry coils of 1000 ohms resistance which are wound on flat bakelite cards. It has been shown in Part I that the effect of the distributed capacity in these coils will add an inductance of magnitude $-cR^2/_3$ to them. For this reason it is important to make shielding the same around the two ratio arms. In our arrangement the flat coils are mounted parallel to each other, symmetrically between three parallel copper plates spaced 1.5 inches apart. The plates are soldered to a copper strip joining the two resistance coils.

3. The Detector.—The detector consists of a two-stage audio transformer coupled amplifier with sensitive telephones. The output from the bridge is fed into the primary winding of the first transformer. By means of the switch S in Fig. 4, the detector may be connected across DB, for recording the balance in the main bridge, or across DE, for the earthing adjustment.

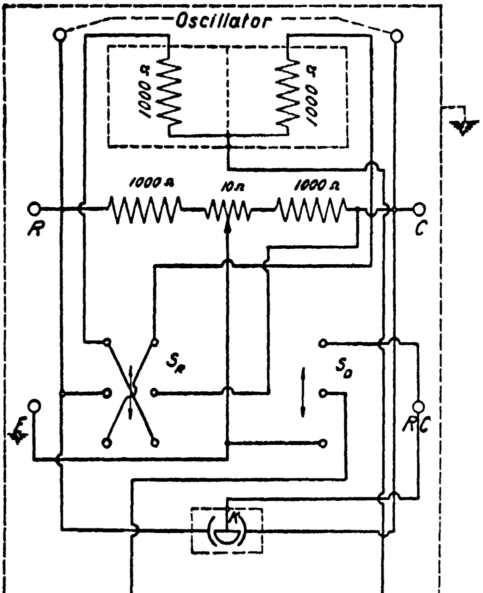

FIG. 5

4. The Earthing Circuit.—The earthing circuit consists of two 1000-ohm bifilar coils (Leeds and Northrup) and a 10-ohm rheostat (W_R) connected as shown in Fig. 4 to the ratio arm extremities, and to ground. In addition, there is a variable air condenser, with a "Vernier" control knob connected between the earth E and the point A (or C, as required).

Various parts of the bridge, including the Oscillator and detector, are shielded from external electrostatic disturbances by the earthed metal shields surrounding them. The various earth points E are all brought together by soldered connections.

The ratio arms, earthing resistances, condensers K, and the reversal and detector switches ("Federal Anti-capacity Switches") are mounted in a grounded copper screened box, as shown diagrammatically in Fig. 5. The top of the box consists of a brass plate in which the terminals are mounted with adequate insulation of transparent bakelite.

5. *Measuring Resistance.*—The resistance standard MB (Fig. 4) consists of three parts. A five-dial box of bifilar coils containing decades of 1/100, 1/10, 1, 10 and 100 ohm steps, is mounted in a shielded box. The screening in the box has a clearance of not less than 2.5 inches from any of the coils or contacts. This spacing proved to be sufficient by actual test to make the effect of the screen capacity on the resistance negligible for our purposes. The control knobs for operating the resistance dials are on the outside of the box, over a brass plate which forms a part of the shielding. They are fastened to the dials through stout bakelite rods. Complete shielding of these lower resistance dials as well as the condenser K (Fig. 5) is of great convenience in entirely eliminating "hand effects" in measurements.

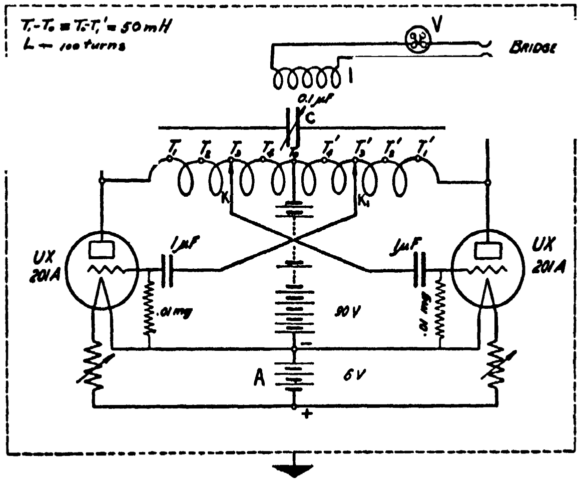

FIG. 6

The effect of temperature on the resistance coils of higher values is not negligible in work requiring readings to 0.01 ohm and is not the same on every coil, so that corrections are not readily made. For this reason we found it desirable to control the temperature of the 1000-ohm step decade and the 10,000-ohm coils. These coils, of a woven type, forming one loop, are made by the Leeds and Northrup Co. They are practically reactance-free, compact and more constant in resistance than the card-wound type, being subject to very slight mechanical strain.

The 1000-ohm decade is mounted in air in a glass jar which is immersed in the metal shielded, oil-filled thermostat which holds the conductivity cell. The 10,000-ohm coils are mounted separately in large test-tubes, which are sufficiently spaced in a wooden frame, and also immersed in the thermostat. The 1000-ohm decade is permanently connected to the resistance box with a stiff heavy wire, avoiding close screening. The required connections in the thermostat, of the cell and measuring resistance are made with short heavy strands of bare copper wire through mercury cups. To avoid appreciable "dead end effects" the 10,000-ohm coils are appended one at a time as needed. Connections to the "bridge box" (Fig. 5) of the various units are made with flexible copper screened stranded wire, the screening being grounded.

6. The Oscillator.—We are indebted to Dr. N. Frank of the Massachusetts Institute of Technology for furnishing us with the specification for an improved type of vacuum tube oscillator, designed by Eccles and Jordan. It consists of a double balance oscillating circuit which has the advantage of giving an alternating current of very good wave form and of more constant frequency and intensity than that generated by the usual type of one-tube oscillator. The circuit is shown in Fig. 6. In varying the magnitude of the capacity C, which is made up of mica condensers which can be thrown in parallel by a system of switches (not shown in Fig. 6), the instrument can be made to give currents of frequencies ranging from about 600 to about 8000 cycles. The filament supply from the 6-volt storage battery A can be thrown on or off by means of a switch located near the bridge, so that the oscillator can be started or stopped conveniently during the measurements. The voltage supplied may be varied by looser coupling of the "pick up" coil L, or by changing the connections of K and K′ to the taps T_1, T_2, T_3 and T_1', T_2', T_3', respectively. It is important to use separate batteries for the oscillator and detector circuits; otherwise, troublesome inductive couplings will occur. A calibrated vacuum thermocouple (V) is included in the apparatus for measuring the current supply to the bridge.

7. Tests and Calibrations.—The resistance box, 100-ohm step decade, and the 10,000-ohm coils were calibrated with direct current against Bureau of Standards resistance standards, by the Carey Foster method, using an auxiliary resistance box. This was done directly with the bridge described above, using a high sensitivity galvanometer, and then checked on a well calibrated direct current Wheatstone bridge. The individual 10,000-ohm coils and various parallel combinations of them were also measured with the shielded bridge, at frequencies of 1000, 2000 and 3000 cycles, and with direct current. The variations from the direct current values obtained were less than 0.002% at 3000 cycles for 10,000 ohms, and entirely negligible at 1000 cycles, or for lower resistances.

The quality of the insulation, which is particularly important for the higher resistance measurements, was tested by connecting a 45-volt battery between the various binding posts, leads and screens, with a sensitive galvanometer in series. It was found to be well over 500 megohms across the important paths. The resistances of the metal contacts, important for the comparatively low resistance measurements, were also tested. A film of vaseline on the terminals and switch points made the resistance of the contacts reproducible and negligible (under 0.01 ohm).

In using the bridge, measurements are made by alternately adjusting R and K for the main balance, and W_R and W_K in the earthing circuit, with the detector in the correct position for each. The unknown and standard are then interchanged by throwing the reversed switch, and the procedure is repeated. The mean of the two readings gives the desired value.

No difficulty is found in making the adjustments, which may be made rapidly with a sensitivity of 0.001% directly without interpolation in measuring resistances of 1000 ohms or over. It is possible to use the bridge for measurements of high precision under conditions in which the use of an unshielded bridge would be quite difficult or impossible due to disturbances from other electrical circuits.

Measurements made with this bridge on the conductance of mixtures of potassium and sodium chlorides have already been reported.

The author wishes to express his gratitude to Dr. D. A. MacInnes for his helpful suggestions both during the progress of the work and in the preparation of the manuscript.

SUMMARY

Part I. The theory of earth and shield capacity effects on alternating currents is discussed, and the results are applied to the Wheatstone bridge. The principles for the design of an electrostatically screened bridge for precision work are developed.

Part II. An alternating current, shielded bridge for precise electrolytic conductance measurements is described.

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A CONDUCTIVITY CELL FOR ELIMINATING ELECTRODE EFFECTS IN MEASUREMENTS OF ELECTROLYTIC CONDUCTANCE

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(Received for publication, December 26, 1930)

The electrolytic conductivity of a solution is ordinarily determined by measuring its resistance, with alternating current, in a cell previously calibrated with a solution having a known specific conductance. The product of this specific conductance and of the corresponding cell resistance is the "cell constant." Values of the specific conductance of other solutions are obtained by dividing the cell constant by the resistance measured in the cell.

Parker[1] has compared measurements on solutions of potassium chloride and hydrochloric acid, obtained in several cells over a range of concentrations. He found that determinations with different cells would lead to somewhat different conductance values. That is, there seemed to be an apparent variation in "cell constant" when the nature or concentration of the measured solutions was varied. Randall and Scott[2] found similar variations on measuring in two cells the conductance of solutions of several substances. These workers found very surprising variations, which in some cases were as high as 0.5%. Such large variations evidently make precision work impossible, and the following study was made with the purpose of eliminating them.

We have made a number of intercomparisons between several cells and have also observed apparent variations in cell constants, although considerably smaller ones than those found by the above investigators. In our work the variations were never greater than 0.2% with bright platinum electrodes, and were always under 0.1% with platinized electrodes. This smaller variation is due, we believe, to the fact that our measurements were made with a bridge[3] which gave readings free from certain sources of error possible in the arrangements these workers used.

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[1] Parker, THIS JOURNAL, 45, 1366, 2020 (1923).

[2] Randall and Scott, *ibid.*, 49, 636 (1927).

Parker has suggested that the variations of cell constants are due to adsorption at the electrode. However, adsorption would be less on bright electrode surfaces, which, as we have just seen, give the largest variations. Therefore, adsorption cannot entirely account for the observations. In addition, the bridge readings with bright electrodes showed fluctuations of a nature suggesting variable polarization effects. Such fluctuations disappeared when the electrodes were lightly platinized, but it is quite possible that such polarization was made constant by this procedure, rather than eliminated.

In this paper is described a cell which was designed for studying means of eliminating any effects at the electrodes which would disturb conductance measurements. The results of test measurements with the cell are also reported. To anticipate the conclusions, these results indicate that cell constant variations disappear if electrode effects are completely eliminated.

The Cell

The cell is shown diagrammatically in Fig. 1. It is provided with two end, or conducting, electrodes O and D, and the three loop, or detector, electrodes a, b and c. The electrodes O and D are hollow cones of platinum foil, made as nearly alike as possible, and a, b and c are loops of platinum wire. All the electrodes are sealed directly to the inner surface of the cell and are very lightly platinized. The cell is constructed from Jena glass 16^{III}, which may be sealed directly to platinum. This glass also has a very low solubility.

Such a cell may be connected into the Wheatstone bridge circuit in a number of different ways. For instance, measurements may be made in the ordinary manner between various electrode pairs. We have used the pairs OD, ab, bc, and ac in this way. This is obviously equivalent to an intercomparison between four ordinary cells. One of the purposes of this investigation was to see whether variations of the cell constant would disappear if similar electrodes were included in two arms of the bridge during measurement. The arrangement is shown in Fig. 2. It was hoped that the disturbing variations would be eliminated if measurements were made with one of the loop electrodes (a, b or c) connected to the detector, electrode O to one end of the current source, and the electrode D to a resistance box in the other arm of the bridge. Thus, for example, the portion of the cell (aO) with the electrode effect from O is balanced by the resistance box in series with the electrode effect from D, and the portion of the cell (aD) K is the condenser for balancing the resistances in the two arms, and the two 1000-ohm coils are the ratio arms. This particular arrangement we have called [a]. [b] and [c] are similar arrangements with the detector connected to (b) or (c) instead of to (a).

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[3] Shedlovsky, THIS JOURNAL, 52, 1793 (1930).

Parker,[1] who has made some excellent determinations of absolute conductance values for potassium chloride solutions, partially corrected for electrode effects by subtracting measurements made in two different cells provided with similar electrodes. He hoped that the electrode effect would be eliminated from the measurements if all determinations were made with the same two cells and the difference between the two readings were always taken as the resistance value. This would be so if the electrodes in the two cells behaved in exactly the same manner in all measurements. The method of making measurements by arrangements [a], [b] and [c] was chosen for obtaining

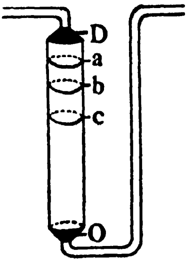

FIG. 1

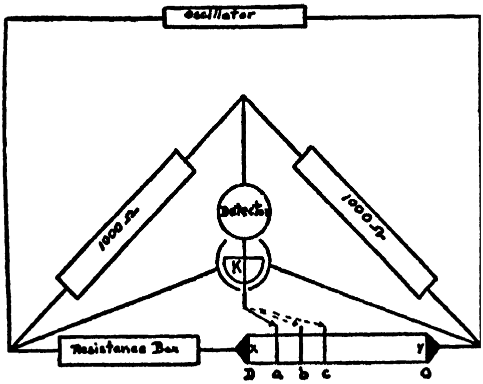

FIG. 2

directly the results given by Parker's two cell scheme. The difference between two of our measurements, ab, ac, bc, using the various pairs of loop electrodes as ordinary cells, corresponds to Parker's actual scheme. In Table II these arrangements are represented by (ac)–(ab) and (ac)–(bc).

Referring once more to Fig. 2, let us call x the effect on the resistance due to the electrode D, and y the effect due to the electrode O. The current density at these electrodes depends on whether the detector is connected to a, b, or c; that is, on the resistance between the detector electrode and the current source terminals, other things being equal. We found that a ten-fold change in current density attained by altering the voltage supplied to the bridge had no effect on the value of a particular measurement, so that it is fair to assume that x and y remain essentially the same whether the detector is connected to a, b or c. Let us call R_a, R_b, R_c the resistance box readings for the arrangements [a], [b] and [c], respectively. Since the loop or detector electrodes draw no current when the bridge is balanced, it seems plausible to assume that there is no effect at such electrodes that influences the readings.

We can write the following equations representing the conditions of balance in our bridge for the arrangements [a], [b] and [c]

$$\begin{array}{ll} [\mathrm{a}] & R_\mathrm{a} + x + \rho_\mathrm{Da} = \rho_\mathrm{oa} + y;\ R_\mathrm{a} = \rho_\mathrm{oa} - \rho_\mathrm{Da} + y - x \\ [\mathrm{b}] & R_\mathrm{b} + x + \rho_\mathrm{Db} = \rho_\mathrm{ob} + y;\ R_\mathrm{b} = \rho_\mathrm{ob} - \rho_\mathrm{Db} + y - x \\ [\mathrm{c}] & R_\mathrm{c} + x + \rho_\mathrm{Dc} = \rho_\mathrm{ac} + y;\ R_\mathrm{c} = \rho_\mathrm{ac} - \rho_\mathrm{Dc} + y - x \end{array}$$

where ρ_Da is the resistance of the solution between D and a, etc. The actual reading observed, for instance R_a, corresponds to the difference between the two fixed portions of the solution Oa and Da, modified by the effects from the electrodes. If $x = y$, this method of measuring conductances is free from these electrode errors. However, if x and y are not equal, the electrode effects can be entirely eliminated by taking the difference between any two of the measurements [a], [b] and [c]. Thus

$$\begin{array}{ll} [\mathrm{a}] - [\mathrm{b}] & R_\mathrm{a} - R_\mathrm{b} = \rho_\mathrm{oa} - \rho_\mathrm{ob} + \rho_\mathrm{Da} + \rho_\mathrm{Db} = 2\rho_\mathrm{ab} \\ [\mathrm{a}] - [\mathrm{c}] & R_\mathrm{a} - R_\mathrm{c} = 2\rho_\mathrm{ac} \\ [\mathrm{b}] - [\mathrm{c}] & R_\mathrm{b} - R_\mathrm{c} = 2\rho_\mathrm{bc} \end{array}$$

Thus, for instance, the difference $R_\mathrm{a} - R_\mathrm{b}$ is equal to twice the resistance of the solution between a and b, without error due to effects at the conducting electrodes.

The Measurements

Measurements were made with the cell described in the various ways discussed above. Four series were carried out at 1000, 2000 and 3000 cycles. First the cell was filled with potassium chloride solution (approx. 0.005 *N*) and then with three different concentrations of hydrochloric acid (approx. 0.001, 0.002 and 0.004 *N*). The cell was immersed in an oil thermostat which maintained a temperature regulation accurately to ±0.001°. The temperature was measured with a platinum resistance thermometer sensitive to about 0.0002°. A shielded bridge, described in another paper,[3] supplied with current from a balanced vacuum valve oscillator, and a detector amplifier was the measuring apparatus. The resistance box used in the measurements was calibrated to 0.01%.

TABLE I

Resistances with (Approx.) 0.005 N Potassium Chloride

	1000 cycles	2000 cycles	3000 cycles
OD	8118.9	8118.9	8118.8
ab	1161.0	1160.6	1160.4
bc	1206.9	1206.6	1206.5
ac	2030.0	2029.6	2029.4
ac − ab	869.0	869.0	869.0
ac − bc	823.1	823.0	822.9
[a]	6064.3	6064.0	6063.6
[b]	4414.6	4414.4	4413.9
[c]	2705.8	2705.5	2705.1
[a] − [b]	1649.7	1649.6	1649.7
[a] − [c]	3358.5	3358.5	3358.5
[b] − [c]	1708.8	1708.9	1708.8

The data are summarized in Tables I and II. The figures shown in Table I represent the resistances corresponding to the various arrangements when the cell was filled with approximately 0.005 *N* potassium

TABLE II

Resistance Ratio Values

	$\frac{\text{Resistance HCl (concn.)}}{\text{Resistance KCl (approx. 0.005 } N)}$				Top value, 1000 cycles middle value, 2000 cycles bottom value, 3000 cycles	
	C = approx. 0.004 N	% error	C = approx. 0.002 N	% error	C = approx. 0.001 N	% error
OD	0.42605	−0.03	0.83440	−0.01	1.6763	−0.02
	.42605	− .03	.83437	− .02	1.6760	− .04
	.42604	− .03	.83437	− .02	1.6756	− .07
ab	....	....	.83437	− .02	1.6755	−. 07
	.42618	+ .01	.83448	....	1.6759	− .05
	.42620	+ .01	.83454	....	1.6760	− .04
bc	....	....	.83429	− .02	1.6760	− .04
	.42616	....	.83441	− .01	1.6762	− .03
	.42619	+ .01	.83448	....	1.6763	− .02
ac	....	....	.83439	− .01	1.6760	− .04
	.42612	− .01	.83445	− .01	1.6762	− .03
	.42613	....	.83439	− .01	1.6764	− .02
ac − ab	....	....	.83437	− .02	1.6767	....
	.42602	− .03	.83437	− .02	1.6772	+ .03
	.42591	− .06	.83414	− .04	1.6768	....
ac − bc	.42604	− .03	.8345	....	1.6761	− .04
	.42604	− .03	.8345	....	1.6761	− .04
			.8342	− .03	1.6765	− .01
[a]	.42599	− .04	.83438	− .01	1.6774	+ .04
	.42601	− .03	.83440	− .01	1.6763	− .02
	.42604	− .03	.83442	− .01	1.6762	− .03
[b]	.42591	− .06	.83437	− .02	1.6762	− .03
	.42593	− .05	.83436	− .02	1.6761	− .04
	.42595	− .05	.83439	− .01	1.6760	− .04
[c]	.42579	− .08	.83425	− .03	1.6760	− .04
	.42584	− .07	.83426	− .03	1.6758	− .05
	.42590	− .06	.83428	− .03	1.6755	− .07
[a] − [b]	.42618	+ .01	.83446	....	1.6767	....
	.42618	+ .01	.83445	....	1.6767	....
	.42618	+ .01	.83448	....	1.6767	....
[a] − [c]	.42615	....	.83450	....	1.6767	....
	.42615	....	.83451	....	1.6767	....
	.42615	....	.83450	....	1.6767	....
[b] − [c]	.42611	− .01	.83456	....	1.6767	....
	.42611	− .01	.83452	....	1.6767	....
	.42610	− .01	.83454	....	1.6767	....
Correct value taken	.42615		.83450		1.6767	

chloride. Table II gives the corresponding resistance ratio, *i. e.*, $R_{\mathrm{HCl}\ (c)}/R_{\mathrm{KCl}\ (0.005\ N,\ \mathrm{approx.})}$ for the three hydrochloric acid solutions measured.

These values are, therefore, proportional to the specific resistance of the three hydrochloric acid solutions as obtained from the various types of measurements, after calibrating with a potassium chloride solution.

It is evident that the arrangements [a] — [b], [a] — [c], [b] — [c], which are free from conducting electrode effects, as shown above, give the most consistent values. These figures show practically no variations with frequency, nor of "cell constant," the average deviation being within 0.01%, which is the precision to which the resistance box is calibrated. We have, therefore, assumed as correct the values obtained by making measurements in this manner. The percentage error of the values obtained from the other arrangements on this basis is indicated in Table II to the nearest 0.01%.

DISCUSSION

Since electrodes O and D were made as nearly alike as possible both in construction and in the subsequent platinizing, we should expect smaller variations in the arrangements [a], [b] and [c] in which these two electrodes are in opposite arms of the bridge than in the ordinary arrangement OD, if the cause of the variations is due to simple adsorption. This does not seem to be generally true, which suggests that besides adsorption there is superimposed another effect. Referring to the equations [a], [b] and [c], the results seem to indicate that x and y may have opposite signs, so that their effect on the measurements may increase instead of decreasing when they appear in opposite branches of the bridge. Such may be the case if there is a definite difference between the rates of building up and decaying of the e. m. f. at the electrode surfaces for the oxidation and reduction parts of the current cycle.

It appears from our results that conductance values, free from errors due to effects at electrodes which tend to change the apparent cell constant, can be obtained with a four electrode cell. The measurements for the arrangements [a] — [b], [a] — [c] and [b] — [c] represent a comparison between three such four electrode cells and lead to consistent conductance values.

Cells of the ordinary type (with two electrodes), which may be more convenient for routine work, can be calibrated by comparison with a four electrode cell.

SUMMARY

1. A multiple electrode cell for studying the elimination of electrode effects in conductance measurements is described.

2. Data obtained with one potassium chloride solution and three hydrochloric acid solutions of different concentrations, using various measuring arrangements of electrodes, are given for three frequencies.

3. The assumption of adsorption at the electrodes does not entirely explain the variations in "cell constant" which are observed.

4. A four electrode arrangement is found to give consistent results at the three frequencies without variations in "cell constant."

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AN APPLICATION OF MOVING BOUNDARIES TO A STUDY OF AQUEOUS MIXTURES OF HYDROGEN CHLORIDE AND POTASSIUM CHLORIDE

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(Received for publication, February 18, 1930)

INTRODUCTION

The purpose of this research was the measurement of the mobilities of the various ion constituents in aqueous mixtures of hydrogen and potassium chlorides. Since the conductances of such solutions do not follow the law of mixtures, it appeared of interest to determine which ion constituents were responsible for these deviations. The moving boundary method was employed in these determinations, boundaries of the rising type being used exclusively. The results, as will be shown below, are also of interest in connection with the recent work on the separation of the rare earth elements by the ionic migration method.[2]

THEORETICAL

The electrolysis of a mixture of hydrogen and potassium chlorides, using, for instance, a cadmium anode as the source of indicating cations, may be represented diagrammatically as follows. The use of the metallic anode in this manner is the method of Cady and Longsworth,[3] though the source of indicating ions may also be a solution of an appropriate electrolyte. Both methods were employed in these experiments. The tube is initially filled throughout with the mixture of uniform composition as shown in Fig. 1. After current has passed for a time, the hydrogen-ion constituent originally in the lower part of the tube will, due to its high mobility, have forged ahead of a portion of the potassium-ion constituent, and there should be the two boundaries, A and B, as shown in Fig. 2. These two boundaries have been observed and their separate progress has been followed quantitatively. The boundary A divides the mixture originally placed in the tube from a region of pure potassium chloride. This boundary travels with a velocity determined by the mobility of the hydrogen-ion constituent and the potential gradient in the mixture.

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[1] National Research Council Fellow.

[2] Kendall and Clarke, *Proc. Nat. Acad. Sci.*, 11, 393 (1925); Kendall and Crittenden, *ibid.*, 9, 75 (1923); Kendall and White, *ibid.*, 10, 458 (1924).

[3] Cady and Longsworth, THIS JOURNAL, 51, 1656 (1929).

The slower boundary B is the usual boundary between the indicating electrolyte formed by the electrochemical oxidation of the cadmium anode and a pure potassium chloride solution. The boundary B travels with a velocity determined by the mobility of the potassium-ion constituent and the potential gradient prevailing at the "adjusted" concentration C_K of the region between the two boundaries. This adjusted concentration can be computed from the expression

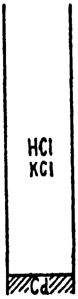

FIG. 1

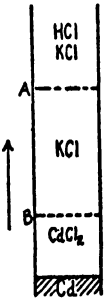

FIG. 2

$$\frac{C'_K}{T'_K} = \frac{C_H}{R_H} + \frac{C_K}{R_K}$$

in which C denotes concentration, T the transference number and R is a function of the ion mobilities. A derivation of this expression follows.

Throughout this treatment the velocity of an ion constituent will be used. This velocity is a measurable quantity. The velocity of an individual ion cannot, at present, be determined experimentally. If the electrolyte is completely dissociated, the velocity of an ion constituent is identical with the individual ion velocity. If incompletely dissociated, the two velocities differ by a factor equal to the degree of dissociation.

Let V_{H} represent the observed velocity of the hydrogen-ion constituent and U_{H} the mobility of this ion constituent, then

$$V_{\mathrm{H}} = U_{\mathrm{H}} \frac{\mathrm{d}E}{\mathrm{d}x}, \text{ and } \Lambda_{\mathrm{H}} = FU_{\mathrm{H}}$$

where Λ_{H} is the conductance of 1 g. equivalent of hydrogen ion. F is the faraday in coulombs if U_{H} is expressed in practical units. The total hydrogen-ion concentration in gram equivalents per milliliter of solution is denoted by C_{H} and the same symbols with appropriate subscripts refer to corresponding quantities for the potassium- and chloride-ion constituents. If A is the (uniform) cross-sectional area in square centimeters of the tube in which electrolysis is taking place, the current flowing in the tube is

$$I = FA(C_{\mathrm{H}}V_{\mathrm{H}} + C_{\mathrm{K}}V_{\mathrm{K}} + C_{\mathrm{Cl}}V_{\mathrm{Cl}}) \tag{1}$$

The electro-neutrality of the solution is expressed by

$$C_{\mathrm{H}} + C_{\mathrm{K}} = C_{\mathrm{Cl}} \tag{2}$$

If x denotes distance along the tube, the quantity of hydrogen ion, Q_{H}, passing through a given cross section at x in Δt seconds is

$$Q_{\mathrm{H}} = (AC_{\mathrm{H}}V_{\mathrm{H}})\Delta t \tag{3}$$

and at $x + \Delta x$

$$Q_{\mathrm{H}} + \Delta Q_{\mathrm{H}} = \left[(AC_{\mathrm{H}}V_{\mathrm{H}}) + \frac{\partial}{\partial x}(AC_{\mathrm{H}}V_{\mathrm{H}})\Delta x\right]\Delta t \tag{4}$$

The change in hydrogen-ion content within the region between x and $x + \Delta x$ is given by the difference between (3) and (4)

$$\Delta Q_{\mathrm{H}} = -\frac{\partial}{\partial x}(AC_{\mathrm{H}}V_{\mathrm{H}})\,\Delta x\,\Delta t$$

Since the corresponding concentration change, ΔC_{H}, is equal to ΔQ_{H} divided by the volume, $A\ \Delta x$, in which the change occurs

$$\Delta Q_{\mathrm{H}} = A\ \Delta x\,\Delta C_{\mathrm{H}} = -\frac{\partial}{\partial x}(AC_{\mathrm{H}}V_{\mathrm{H}})\,\Delta x\,\Delta t \tag{5}$$

In the limit, Equation 5 becomes

$$A\frac{\partial C_H}{\partial t} + \frac{\partial}{\partial x}(AC_HV_H) = 0 \tag{6}$$

and similarly for the potassium-ion constituent

$$A\frac{\partial C_K}{\partial t} + \frac{\partial}{\partial t}(AC_KV_K) = 0 \tag{7}$$

Multiply (6) by $F(V_H + V_{Cl})/V_H$ and (7) by $F(V_K + V_{Cl})/V_K$, assuming the ratios $(V_H + V_{Cl})/V_H$ and $(V_K + V_{Cl})/V_K$ to be independent of the concentration, and add the resulting equations

$$AF\frac{V_H + V_{Cl}}{V_H}\frac{\partial C_H}{\partial t} + AF\frac{V_K + V_{Cl}}{V_K}\frac{\partial C_K}{\partial t} + \frac{\partial}{\partial x}[FAC_H(V_H + V_{Cl})] + \frac{\partial}{\partial x}[FAC_K(V_K + V_{Cl})] = 0$$

Collecting terms and setting $1/R_H = (V_H + V_{Cl})/V_H$ and $1/R_K = (V_K + V_{Cl})/V_K$,[4]

$$\frac{AF}{R_H}\frac{\partial C_H}{\partial t} + \frac{AF}{R_K}\frac{\partial C_K}{\partial t} + \frac{\partial}{\partial x}[FA(C_HV_H + C_KV_K + C_HV_{Cl} + C_KV_{Cl})] = 0 \tag{8}$$

From (1) and (2) it is seen that the bracketed expression in (8) is the current I, and since this is uniform throughout the tube

$$\frac{\partial I}{\partial x} = 0$$

The resulting differential equation can then be integrated to give

$$\frac{C_H}{R_H} + \frac{C_K}{R_K} = \text{constant} + f(x) \tag{9}$$

This corresponds to, but is not identical with, the "beharrlichen" function of Kohlrausch.[5] For any given value of x it retains its initial value (neglecting diffusion and convection) throughout electrolysis. If the composition of the solution originally present in the tube is uniform, $f(x)$ also becomes constant. Application of this function,

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[4] The symbol R is identical with the transference number for a simple binary electrolyte but is not the transference number of an ion constituent in a mixture. In the latter case the transference number is given by a more complicated expression; see D. A. MacInnes, THIS JOURNAL, 47, 1922 (1925).

[5] Kohlrausch, *Ann. Physik*, 62, 209 (1897).

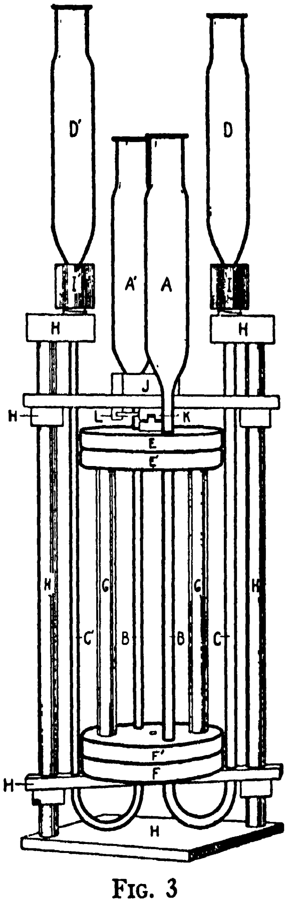

Fig. 3

which will henceforth be termed the "regulating" function, to the experiments described here yields the relation

$$\frac{C_{\mathrm{H}}}{R_{\mathrm{H}}} + \frac{C_{\mathrm{K}}}{R_{\mathrm{K}}} + \frac{C'_{\mathrm{K}}}{T'_{\mathrm{K}}} = \text{constant} \tag{10}$$

The primed quantities in (10) refer to the region of pure potassium chloride solution between the two boundaries, A and B, Fig. 2.

In connection with this regulating function it should be pointed out that the derivations given by Kohlrausch[5] and von Laue[6] are based on the Arrhenius theory of solutions of electrolytes with the attendant assumption of constant ion mobility. Experimentally, the mobility of an ion constituent varies more rapidly with the concentration than does a ratio of these mobilities. Consequently the assumption of R independent of concentration is a more limited assumption than the constancy of the individual mobilities. Otherwise the treatment given here is similar to that of von Laue and the generalized Equation 5, page 332, in his development is identical in form with Equation 9.

The Moving Boundary Apparatus

The cell used for these measurements in which the source of indicator ion is a solution is shown in Fig. 3. The shearing mechanism of MacInnes and Brighton[7] was utilized for forming the boundary. The plate glass disks EE′ and FF′ are etched in the manner described by these authors and the electrode chambers AA′D and D′ are designed according to their recommendations. Tubes B and B′ are graduated[8] and calibrated tubes in which the motion of the boundary is observed. The system ABCD is quite independent of the system A′B′C′D′, the tube B having an average cross-sectional area about twice that of B′. Only one of these tube systems is used in a determination. The glass disks E′ and F′, together with the brass supporting rods GG and the tubes BB′, constitute an independent unit of the cell and may be removed for cleaning. By clamping this central unit to the lower disk F and turning E over E′, boundaries may be formed which can reach the graduations by descending. On the other hand, clamping the central unit to the upper disk E and turning F′ over F gives rise to boundaries that can be observed if they ascend the tube. The cell may thus be adapted for the study of either rising or falling boundaries, though all observations made in this paper were on the first mentioned type.

The parts H are of brass and constitute the supporting framework for the glass parts of the cell which are traced in heavier lines. I and I′ are threaded bakelite couplings to permit removal of the electrode chambers D and D′ for cleaning.

The manipulation using the tube system ABCD and observing a rising boundary is as follows. The plates E and E′ are lubricated and clamped together with the tube B and chamber A in alignment. This unit is then filled with the solution whose transference number is to be determined. The tube C and chamber D are filled with solution of indicator electrolyte. Silver-silver chloride electrodes are introduced into the electrode chambers and, if necessary, a few milliliters of a more concentrated solution introduced around these electrodes. The disks F and F′

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[6] M. von Laue, *Z. anorg. Chem.*, **93**, 329 (1915).

[7] MacInnes and Brighton, THIS JOURNAL, **47**, 994 (1925).

[8] MacInnes, Cowperthwaite and Huang, *ibid.*, **49**, 1710 (1927).

are then lubricated and pressed together with the protruding drops of solution from B and C in their respective recesses in the opposite disk, as described by MacInnes and Brighton.[7] The cell is then placed in a vibration-free thermostat and allowed to come to thermal equilibrium. Potential is then applied and the circuit tested to see that no current is flowing. The boundary is next formed in the following manner.

A coil spring J of phosphor bronze furnishes power for shearing the plate glass disks in initially forming the boundary. The brass coupling K transmits this power to the disk, also permitting the removal of the disk, E, together with the electrode chambers AA′ which are sealed into it, for cleaning. A release mechanism L is operated by a flexible camera shutter cable (not shown in the diagram). This arrangement permits the turning of the plates and the formation of the boundary without starting vibration. The desirability of such an arrangement has been discussed by MacInnes, Cowperthwaite and Shedlovsky.[9] Upon the release of the spring tension, due to the viscosity of the lubricant between the plates, the plate F′, together with the cell units rigidly connected to it, smoothly turns about its center until tube B is exactly over tube C. The boundary is thus formed and starts to rise immediately, since the electrical circuit is now complete.

For rising boundaries where, according to the method described by Cady and Longsworth,[3] a metallic anode is used as the source of indicator ions, a similar cell was employed. It differs from the one just described in that a cylindrical recess was ground in the lower plate glass disk in which a small disk of anode metal was placed, and also in that no shearing mechanism is necessary.

The current through the cell was maintained constant throughout a determination by means of an improved constant current regulator.[10] This regulator is a modification of the apparatus used by MacInnes, Cowperthwaite and Blanchard,[11] modified so as to give a constant current in a circuit in which both increases and decreases of resistance may occur.

Materials and Purification.—The potassium chloride was recrystallized twice from conductivity water and fused in a platinum crucible. The hydrochloric acid was a pure product, diluted to constant boiling strength and distilled, the end fractions being rejected as recommended by Foulk and Hollingsworth.[12] The concentration of this constant boiling acid was interpolated from the data of these authors.

The materials used as indicators were treated as follows. The potassium iodate was recrystallized from water and dried over phosphorus pentoxide *in vacuo*. The sodium salt of iodeosin (tetra-iodofluorescein) was especially prepared by the makers and was further purified by four recrystallizations from water in this Laboratory. The cadmium metal and iodic acid were of a good grade and were not further purified.

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[9] MacInnes, Cowperthwaite and Shedlovsky, THIS JOURNAL, **51**, 2671 (1929).

[10] Longsworth and MacInnes, *J. Opt. Soc. Am.*, **19**, 50 (1929).

[11] MacInnes, Cowperthwaite and Blanchard, THIS JOURNAL, **48**, 1909 (1926).

[12] Foulk and Hollingsworth, *ibid.*, **45**, 1220 (1923).

Conductivity Measurements.—The conductances of the solutions were measured on the shielded bridge developed by Dr. Theodore Shedlovsky of these Laboratories. Since this bridge has been described in a separate communication,[13] the following brief notes will suffice here. Pipet cells of the Washburn type were calibrated with 0.1 demal potassium chloride solution according to the recommendations of Parker and Parker.[14] An audion oscillator operating at a frequency of 2000 cycles supplied the bridge current, which was amplified by two stages of audio frequency amplification before passing to the telephones. The bridge was of the direct reading type with equal ratio arms. The thermostat in which the conductivity cells were immersed was filled with oil. The temperature was maintained constant at 25 ± 0.002°. The temperature was determined by means of a carefully calibrated platinum resistance thermometer.

Rising Boundaries.—Since MacInnes, Cowperthwaite and Huang,[8] working with rising boundaries, observed convection currents due to heating, it seemed necessary to investigate this effect in detail. Their experiment with a rising boundary between potassium chloride and potassium permanganate, used as indicator electrolyte, was repeated and the same phenomena were observed. These were a diffuse color margin, and a "chimney" tinged with the purple color of the permanganate ion in the potassium chloride solution above the boundary. However, I have found that when the much heavier and less mobile iodeosin ion is substituted for the permanganate ion, an extremely sharp margin results with no trace of the intense red color of this ion in the colorless solution in advance of the boundary. The iodeosin ion has been used as an indicator by Franklin and Cady.[15]

If the mobilities of the chloride- and permanganate-ion constituents, and also the densities of the two solutions under the conditions of the experiment, are compared, it appears quite likely that the differences in these properties are not great enough to permit the development of an effective margin. An effective moving boundary results when the restoring effect due to the potential gradient change at the boundary is strong enough to overcome the disturbing effects of convection, diffusion and, possibly, electro-osmosis. The restoring effect has been described in a paper by MacInnes and Cowperthwaite.[16]

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[13] Shedlovsky, This Journal, 52, 1793 (1930).

[14] Parker and Parker, *ibid.*, 46, 312 (1924).

[15] Franklin and Cady, *ibid.*, 26, 499 (1904).

With rising boundaries mixing due to convection will be greater than with falling boundaries because with the former the hotter solution is underneath, an arrangement which tends to produce circulation in the cooler solution. Due to this greater tendency to mix, the restoring effect must, therefore, be greater in rising boundaries. However, mixing of the two solutions at the boundary will be decreased if there is a large difference in the density of those solutions. The choice of the proper indicator will, therefore, be a matter of experiment. For the determination of the anion mobilities in the solutions studied in this investigation, iodic acid and potassium iodate were used as the indicating electrolytes. The low mobility of the iodate-ion constituent and the high density of the solutions of its salts apparently eliminated the troubles, mentioned above, due to heating and convection.

One important advantage of rising boundaries over the descending type is the wider concentration range throughout which there is automatic concentration adjustment of the indicator solution. It is, therefore, not so necessary to make determinations at a series of indicator electrolyte concentrations as when working with falling boundaries. The data of Table I show the effect of indicator-ion

[16] MacInnes and Cowperthwaite, *Proc. Nat. Acad. Sci.*, **15**, 18 (1929). These authors find experimentally that upon interruption of the current the two solutions forming the boundary begin to diffuse into each other. However, though the current may be interrupted for as much as thirty minutes, upon closing the circuit again the boundary reforms and soon recovers its original sharpness. After reformation and during the period between the reclosing of the circuit and the return to its original sharpness, the boundary moves at the correct velocity. The authors explain this restoring effect of the current as due to the change of potential gradient occurring at the boundary. The lower specific conductance of the indicator solution means a steeper potential gradient in this region. If a more mobile ion in front of the boundary diffuses into the indicator region, it will be subject to a steeper potential slope and will forge ahead of the surrounding less mobile indicator ions until it has regained the boundary. Conversely, if an indicator ion diffuses across the boundary into the region of lower potential slope prevailing in the solution under observation, it will, of necessity, lag behind until it has fallen back into the indicator region. This restoring mechanism of the potential gradient change at the boundary tends to keep the two ion constituents separated and the boundary sharp.

concentration upon the observed anion transference number of potassium chloride at 0.1 normal or 0.1 g. equivalent per liter of solution. This unit of concentration differs from the one defined in the theoretical part by a constant factor of 10^{-3}, that is, for 0.1 normal potassium chloride, $C_{\mathrm{KCl}} = 10^{-4}$. From Table I the transference number is seen to be independent of the concentration of the indicator solution of potassium iodate if the latter is between about 0.06 and 0.15 normal. A normality of about 0.06 is the value required by the "regulating" function applied to this simple boundary. To understand why concentration adjustment over such a wide range is not attained in the case of falling boundaries, let us consider the following comparison of the two methods.

TABLE I

The Effect of the Indicator Concentration on the Observed Transference Number of Potassium Chloride

Normality		dE/dx volts/cm.	Ta
KCl	KIO_3		
0.1	0.05	3.086	[a]
.1	.066	3.692	0.5100
.1	.075	3.995	.5103
.1	.1	3.086	.5100
.1	.1	4.298	.5101
.1	.15	4.298	.5106
.1	.2	4.298	.5120

[a] Margin quite distorted and computed value of T_a changed from 0.532 at beginning of run to 0.513 at the close.

If a margin is formed between 0.1 normal potassuim iodate and 0.1 normal potassium chloride, Fig. 4 represents the initial condition. On passage of a current the boundary will rise and after some time Fig. 5 will represent the new conditions in the tube. That is, a concentration gradient between 0.1 normal and 0.06 normal potassium iodate will remain at C. Since the relation of the densities d are $d_{0.1\,N\,\mathrm{KCl}} < d_{0.06\,N\,\mathrm{KIO_3}} < d_{0.1\,N\,\mathrm{KIO_3}}$, the lighter solution is on top and this distribution will not be disturbed by gravity. On the other hand, consider an analogous case for falling boundaries where the boundary is initially formed between 0.1 normal potassium chloride and 0.1 normal lithium chloride, Fig. 6. After the boundary has fallen some distance, Fig. 7 would represent conditions were it not for the fact that a denser solution of lithium chloride would thus be left on top. These two solutions of lithium chloride would consequently tend to mix and destroy the concentration adjustment as fast as it was made. Both rising and falling boundaries are found, however, to give erroneous results when the indicator-ion concentration is initially too low.

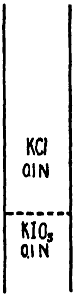

FIG. 4

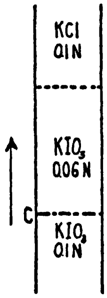

FIG. 5

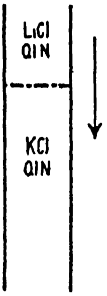

FIG. 6

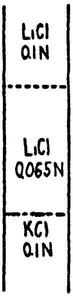

FIG. 7

Experimental Results

Table II contains the results of transference measurements on the anion constituents. The chloride-ion concentration in all mixtures was maintained at 0.1 normal. The composition of the mixtures will be indicated by the ratio y of hydrogen-ion content to the chloride-ion content, that is, $y = C_H/C_{Cl}$ and $(1 - y) = C_K/C_{Cl}$. The indicator solutions for the anion constituents of the mixtures were made up to a total iodate-ion concentration of 0.1 normal, the ratio of hydrogen to potassium-ion content in these solutions being the same as that of the solution for which they were to serve as indicators.

The value of Λ_{Cl} marked with an asterisk is computed from the value of $T_H = 0.8305$ recorded in Table III. This value is given preference since it is the mean of a larger number of determinations. Comparison of the results for the solutions of pure hydrogen chloride and pure potassium chloride as given in Tables II, III and V shows that $T_c + T_a = 0.9998$ for the former and 0.9999 for the latter. The deviation from the theoretical value of unity is within the experimental error.

From Table II it is seen that Λ_{Cl} from 0.1 normal hydrogen chloride is slightly higher than from 0.1 normal potassium chloride. This is at variance with the constant value of Λ_{Cl} from these two salts found by MacInnes and Smith.[17] The author, however, has determined the chloride-ion conductance from a series of uni-univalent chlorides over the concentration range from 0.1 to 0.02 normal. The results deviate by a few tenths of one per cent. from the constancy found by MacInnes and co-workers at the higher concentration. These deviations from constancy tend to disappear, however, as the concentration is reduced. The chloride-ion conductance from these uni-univalent chlorides seems to be a function of the conductance of the co-ion.

TABLE II

Chloride-Ion Conductance in Mixtures of Hydrochloric Acid and Potassium Chloride at a Total Concentration of 0.1 N and 25°

Solution C_H/C_{Cl}	T_{Cl}	Λ	Λ_{Cl}	Λ_{Cl} (calcd.)
1.00	0.1693	391.28	66.32*	...
0.75	.2041	324.31	66.19	66.18
.50	.2560	258.07	66.07	66.03
.25	.3414	192.83	65.83	65.89
.00	.5101	128.89	65.75	...

The values of Λ_{Cl} quoted in the last column of Table II were computed on the basis of linear variation between the chloride-ion conductances in pure hydrogen chloride and pure potassium chloride solutions. The conductance of the chloride-ion constituent in the mixtures is thus seen to vary linearly between its values in the pure solutions. This is in complete agreement with the findings of MacInnes, Cowperthwaite and Shedlovsky[9] on mixtures of sodium chloride and potassium chloride. These authors find that though the conductances of the mixtures deviate from additivity, the conductance of the chloride ion remains constant. In other words, the mobility of the common ion constituent is not the cause for the deviation from additivity of the conductances of mixtures of this valence type.

Table III contains the results of observations on the leading boundary A of Fig. 2. As explained in a preceding section, this boundary is that which arises between the mixture of acid and salt and the region of pure potassium chloride. This boundary was visible as a refraction margin in only one of the mixtures ($y = 0.75$), where it was faint but sharp. In the other two mixtures the progress of this boundary was ascertained by the color change induced in a trace of methyl violet introduced into the solution. In Table IV are given data showing that the presence of the trace of dye did not materially affect the progress of the boundary in the one mixture where direct comparison could be made. V'_A and V'_B of Table IV are quantities proportional to the velocities of the boundaries A and B, respectively, Fig. 2.

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[17] MacInnes and Smith, THIS JOURNAL, 47, 1009 (1925).

TABLE III

Hydrogen-Conductance in Mixtures of Hydrochloric Acid and Potassium Chloride at a Total Concentration of 0.1 N and 25°

Solution C_H/C_{Cl}	Λ_H	$y\Lambda_H$	$T_H = y\,\Lambda_H/\Lambda$
1.00	324.96	324.96	0.8305
0.75	322.4	241.8	.7456
.50	319.9	159.9_5	.6198
.25	316.9	79.2_3	.4109
.00	...	0.00	.0000

The equivalent conductance Λ_K of the potassium-ion constituent in the mixtures may be computed by the relation

$$(1 - y)\,\Lambda_K = \Lambda - \Lambda_{Cl} - y\Lambda_H$$

These values, together with those of the other ion constituents, are collected in Table V.

The values marked with an asterisk are extrapolated, assuming linear variation with the concentration of that constituent. They represent, respectively, the equivalent conductance of vanishingly small quantities of hydrogen ion in 0.1 normal potassium chloride and of potassium ion in 0.1 normal hydrochloric acid.

As the experimental work here presented was being completed, Bennewitz, Wagner and Kuchler[18] reported an extension of the

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[18] Bennewitz, Wagner and Kuchler, *Physik. Z.*, 30, 623 (1929).

Onsager[19] equations for the conductance of strong binary electrolytes to the case of a ternary ion mixture in which one ion constituent is present in very small quantities. The theory, as developed, is valid only at high dilutions where the equivalent conductance varies as the square root of the concentration. To extrapolate from this region of validity to 0.1 normal solutions can yield predictions of only a qualitative nature. In any event, their theory predicts that the equivalent conductance of vanishingly small quantities of hydrogen ion in 0.1 normal potassium chloride will be less than in 0.1 normal hydrogen chloride, while the conductance of the potassium ion will be slightly increased in going from 0.1 normal potassium chloride to 0.1 normal hydrogen chloride. Reference to Table V shows that the extrapolated ion conductances are in qualitative agreement with their theory.

TABLE IV

Effect of Presence of Methyl Violet on the Boundary Velocities in the Mixture 0.075 N HCl–0.025 N KCl

Experiment No.	Dye present		←—— Dye absent ——→		
	1	2	3	4	5
V'_A	9954	9952	9952	. .	. .
V'_B	7055	7055	7063	7058	7057

TABLE V

Conductance of the Ion Constituents of Mixtures of Hydrochloric Acid and Potassium Chloride at a Total Concentration of 0.1 N and 25°

Solution C_H/C_{Cl}	$(1 - y)\ \Lambda_K$	T_K	Λ_H	Λ_K	Λ_{Cl}
1.00	0.00	0.0000	324.96	65.5*	66.32
0.75	16.3	.0503	322.4	65.2	66.19
.50	32.0_5	.1242	319.9	64.1	66.07
.25	47.8	.2477	316.9	63.7	65.83
.00	63.14	.4898	314.0*	63.14	65.75

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[19] Onsager, *Physik. Z.*, **27**, 388 (1926); **28**, 277 (1927).

Concentration Changes and Test of the Regulating Function

Referring once more to Fig. 2, if the theoretical treatment leading to Equation 10

$$\frac{C_H}{R_H} + \frac{C_K}{R_K} = \frac{C'_K}{T'_K}$$

is valid, this equation provides a means for computing the "adjusted" concentration C'_K from the ionic mobility relationships across the boundary A and the known concentrations above this leading boundary. Since the leading solution of boundary B is the indicator solution of boundary A, this adjusted concentration C'_K can also be computed independently from observations on the motion of boundary B. If the results of the two methods agree, this is evidence for the correctness, of Equation 10, which is an expression of the "regulating" function.

For a boundary between two simple solutions such as those meeting at the boundary B, there is the relation

$$T = \frac{VCF}{It} \tag{11}$$

In this equation, T is the transference number, V the volume swept through by the boundary in t seconds and the other terms represent, as before, the concentration, the faraday and the current. It is evident, therefore, that if T is known, the concentration C may be found. From the observed velocity of the boundary B and the independently determined transference number of potassium chloride over the range of concentrations involved, C'_K has been computed by means of Equation 11. If the transference number changed very rapidly with the concentration, such a computation would require a series of approximations. For potassium chloride solutions, however, the transference number varies so slightly with concentration that one approximation is sufficient. The concentrations thus obtained are given in Col. 4 of Table VI, which also includes the data necessary for these computations. These data are the values of VF/It determined experimentally from the velocity of B, and the independently determined values of T'_K for pure potassium chloride solutions.

For comparison the values of C'_K (2) calculated by means of Equation 10 are recorded in Col. 7 of Table VII. The values of

R_H and R_K used in this calculation and recorded in Cols. 2 and 4 are computed from the data of Table V. Reference to the values of the ion conductances given in Table V indicates, however, that the velocity ratio, $V_K(V_K + V_{Cl}) = \Lambda_K/(\Lambda_K + \Lambda_{Cl})$, is slightly different on the two sides of the boundary A. Thus the assumption as to the constancy of this ratio is only an approximation, but a rather close one in this case. The values of C'_K (3) recorded in the last column of Table VII are computed assuming the ratio R_K to remain constant on the two sides of the boundary A and equal to T_K, the ratio or cation transference number in pure potassium chloride at the "adjusted" concentration C_K. The independently computed values (1) are copied from Table VI to facilitate comparison.

TABLE VI

Determination of the "Adjusted" Concentration of the Region oj Pure Potassium Chloride Solution Formed on Electrolysis of Mixtures of Hydrochloric Acid and Potassium Chloride

Solution C_H/C_{Cl}	VF/It	T'_K	$C'_K \times 10^6$
0.75	7065	0.4897	69.31
.50	6154	.4898	79.59
.25	5452	.4899	89.86

TABLE VII

Test of the Regulating Function for Mixtures of Hydrochloric Acid and Potassium Chloride

$C_H \times 10^6$	R_H	$C_K \times 10^6$	R_K	T'_K	$C'_K \times 10^6$ (1)	$C'_K \times 10^6$ (2)	$C'_K \times 10^6$ (3)
75	0.8296	25	0.4962	0.4897	69.31	68.93	69.27
50	.8288	50	.4923	.4898	79.59	79.29	79.55
25	.8281	75	.4919	.4899	89.86	89.49	89.79

In Table VII the agreement between the independently computed values for the adjusted concentration of the region of pure potassium chloride solution between the two boundaries is quantitative evidence for the validity of the regulating function (Equation 10) under the conditions of the experiments. Furthermore, in this region between the two boundaries a portion of the potassium-ion constituent has been concentrated free from the more mobile hydrogen ion present in the original mixture. Though the differences in the mobilities of the two cation constituents of these mixtures are very large, it is probable that the same regulating mechanism is operating in cases where the ion constituents have more nearly the same mobilities, as in mixtures of the rare earths.

DISCUSSION OF RESULTS

All experimental values quoted in this paper are the mean of several closely agreeing determinations. The boundary velocities have been corrected for the volume changes as described by Lewis.[20] The apparent equivalent volume was used in making these corrections, this being assumed additive for the mixtures.

TABLE VIII

Comparison of the Transference Numbers of the Solutions of the Pure Salts as Obtained by Different Investigators

Solution	Dennison and Steele, 18°	MacInnes, *et al.*, 25°	This paper, 25°
0.1 *N* HCl	0.835	0.8320	0.8305
0.1 *N* KCl	.492	.4920	.4899

Bennewitz, Wagner and Kuchler[18] report measurements by the Hittorf method on several mixtures at a total concentration of 0.1 normal and at 18°. The only solution where comparison is possible, however, is that which is 0.05 normal to both hydrogen and potassium chlorides. For this solution at 18°, they obtain $T_H = 0.622$, $T_K = 0.124$ and $T_{Cl} = 0.254$. At 25°, the values recorded by the author are $T_H = 0.6198$, $T_K = 0.1242$ and $T_{Cl} = 0.2560$.

With regard to solutions of the pure electrolytes, reference has already been made to the work of MacInnes and co-workers. Denison and Steele,[21] using lithium chloride and potassium acetate as indicators obtained 0.492 as the cation transference number of 0.1 normal potassium chloride at 18°. Using potassium chloride and iodic acid as indicators for the ions of hydrogen chloride, they obtained a cation transference number of 0.835 for this electrolyte at 0.1 normal and 18°. A comparison of the published values by the moving boundary method is given in Table VIII. The agreement is only fair. The data of this paper fulfil the following conditions, however. (1) They are independent of the potential gradient within wide limits. (2) They are independent of the nature and concentration of the indicator ion within certain limits. (3) They are very reproducible. (4) The sum of the anion and cation transference numbers is very nearly unity.

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[20] Lewis, THIS JOURNAL, 32, 862 (1910).

[21] Denison and Steele, *Z. physik. Chem.*, 57, 110 (1907).

The author wishes to express appreciation to Dr. D. A. MacInnes of these laboratories for the inspiring supervision of this work, to Dr. Theodore Shedlovsky for his coöperation in the determination of the conductivities of the solutions, and to Professor H. P. Cady of the University of Kansas for many helpful suggestions.

SUMMARY

Ion mobility relations and concentration changes on electrolysis in mixtures of hydrogen and potassium chlorides at a total chloride-ion concentration of 0.1 normal have been studied by means of the moving boundary method, using rising boundaries.

Two cation boundaries are formed simultaneously and proceed at different rates. The velocity of the leading boundary yields the mobility of the hydrogen-ion constituent in the mixture. A portion of the less mobile potassium-ion constituent logs behind the hydrogen-ion constituent and gives rise to a second boundary whose velocity yields information as to the concentration adjustment occurring in the region of the solution which contains pure potassium chloride between the two boundaries. From data on both boundaries it is shown that within this latter region the hydrogen ion has been quantitatively separated from the potassium ion and that the concentration of the potassium chloride residue is accurately determined by the "regulating" function of Kohlrausch,[6] a derivation of which is given.

It is shown experimentally that the mobility of the chloride-ion constituent varies linearly between its slightly different values in 0.1 normal hydrogen chloride and 0.1 normal potassium chloride.

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DIETHYLBARBITURATE BUFFER

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In the series of buffers now used, there is no other buffer system to cover the range around pH = 8 with a sufficient reliability except for the borate buffer of Sörensen. This buffer has, however, certain disadvantages which are strongly felt in practical use. The borate buffer cannot be used in compounds containing two or more hydroxyl groups, such as polyphenols, polyhydric alcohols, and especially carbohydrates of any kind. These substances combine with boric acid to form complex acids of an appreciable strength. Not only is the pH of the borate buffer perceptibly shifted in the presence of those substances but, what is worse, those substances themselves are no longer present in their original state in contact with the borate buffer. This disadvantage was encountered on the occasion of the investigation of the effect of pH on the activity of all carbohydrate-splitting enzymes and on the measurement of oxidation-reduction potentials of certain dyestuffs containing two hydroxyl groups, to be described in a subsequent paper. This gap in the buffer series is due to the fact that practically no acid was known with a dissociation constant around 10^{-8}. Recently Kolthoff (1) found a dissociation constant of approximately 10^{-8} for diethylbarbituric acid (veronal). So it seemed likely to me that this acid might be useful as a buffer for the wanted range. This turned out to be true. The following data, calculated from the experimental calibration with the hydrogen electrode, will suffice for practical purposes.

10.30 gm. of sodium diethylbarbiturate (veronal sodium) are dissolved in CO_2-free water to a volume of 500 cc. The quality of the commercial preparation was satisfactory without recrystallization. It may be dried at 100° but loses only traces of moisture by this process. 10 cc. of this solution when titrated against 0.1 M HCl should use up accurately 10 cc. of the acid to the turning point of methyl red. If the veronal solution turns out to be a little weaker (say 1 per cent) a correction for the number of cc. indicated in the first column of Table I may be applied. The error, on neglecting this correction, however is practically negligible except for the range of pH < 7.2. When n cc. of this stock solution of veronal sodium (first column of Table I) are mixed with $(10 - n)$ cc. of 0.1 M HCl, the pH indicated in the second column

TABLE I

Veronal Buffer

When n cc. of 0.1 M veronal sodium are mixed with $10 - n$ cc. of 0.1 M HCl, the following pH values are obtained.

n	pH	n	pH	n	pH
(5.10)	(6.40)	6.15	7.60	9.08	8.80
(5.14)	(6.60)	6.62	7.80	9.36	9.00
5.22	6.80	7.16	8.00	9.52	9.20
5.36	7.00	7.69	8.20	9.74	9.40
5.54	7.20	8.23	8.40	9.85	9.60
5.81	7.40	8.71	8.60	(9.93)	(9.80)

The values in parentheses cannot be considered as accurately reproducible with respect to pH.

is obtained. The potentiometric calibration has been made for 25° ± 0.05°, the pH of standard acetate being considered 4.62.

This buffer has a satisfactory buffer capacity between pH 7.0 to pH 9.4, and so covers not only the gap between the phosphate and the glycocoll buffers but it also duplicates the pH range of particular physiological importance around 7.4 for which only the phosphate buffer was available till now, except for the occasional and restricted use of the cacodylate buffer (2). Though the pH range around 7.4 does not lie quite in the region of the maximum buffering capacity of this buffer, which is pH 8 to pH 9, it can be safely used between pH 7 and pH 8 with carefully prepared stock solutions.

SUMMARY

Veronal is recommended as a buffer covering a range from pH 6.8 to pH 9.6. It replaces not only the borate buffer which is undesirable in many cases but also duplicates a part of the phosphate buffer range, especially around the physiologically important pH 7.4.

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SOME REDOX INDICATORS

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(Received for publication, April 23, 1930)

The colorimetric determination of oxidation-reduction potentials by means of dyestuff indicators has been placed on a sound theoretical foundation by Clark and his associates (1). Some more indicators have been recently measured by Rapkine, Struyk, and Wurmser (2), furthermore by Vellinger (3). The practical application of this principle can be improved upon only by finding new indicators, in the first place to enlarge the range of redox potential accessible to a colorimetric determination especially towards the negative (hydrogen) side of the potential scale, and secondly to duplicate the dyes now available with others covering the same potential range, but of different chemical constitution. The agreement of a potential determined by several indicators of different chemical constitution is the best evidence for the reliability of the indicator method.

In the present paper three dyestuffs are to be described which may be useful for the indicator method. All three have been known for a long time but were never used for any scientific purpose. They form entirely reversible redox systems and give true homogeneous non-colloidal solutions in the ordinary buffers in a concentration sufficient for any colorimetric use.[1] These dyes turned out to be practically pure as delivered from the factory in so far as they contained no other reducible substance, at least none which would cause an overlapping of the titration curve or show a confusing color effect. As the course of the titration curves is strictly that of simple reversible systems we may also exclude the possibility that any of these dyes might be a mixture of different chemical individuals with overlapping curves. These three dyes are the following.

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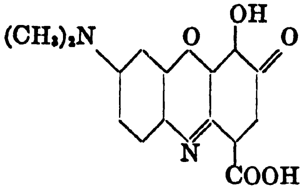
[1] The authors are indebted to the I. G. Aktiengesellschaft Farbenindustrie, Germany, for these dyes. They were selected from a large collection of dyes placed at our disposal according to our suggestions from the stock of the scientific laboratories of this company. We are especially indebted to the liberality of Dr. H. Benda and Dr. I. Peiser. The dyestuffs will be distributed by the Winthrop Chemical Company, Incorporated, 170 Varick Street, New York, N. Y. These will be in accurate agreement with the formulæ shown in this paper.

1. Gallocyanine[3] (Köchlin, 1881).



This is an oxazine and is of amphoteric nature. It is easily soluble at alkaline and at strongly acid reaction, but very slightly soluble between pH 5.5 and 3.5. The solutions have a red-violet color at pH < 4, blue from pH 5.5 to about 8, and red-violet at pH > 8. The insoluble form is obviously that of the isoelectric compound. The red form at strongly acid reaction is the positive ion, ionized at the dimethylamino group, the blue state is the monovalent ion due to the carboxyl group, and the red form at pH > 8 is a bivalent anion due to the hydroxyl group. The reduced form contains an additional hydroxyl group, and, being a polyphenol, it reacts with boric acid to give a complex acid. This prevents the use of the borate buffer in calibrating the constants of the dye. That range of pH (around 8) which is covered only by the borate buffer happens to be a very important range for the characterization of the dye, as can be seen from the curve in Fig. 3. In order to fill the gap due to this deficiency of the borate buffer we used successfully the veronal buffer recently described (6).

From the properties mentioned above it is easily understood that gallocyanine is very slightly soluble in pure water. The dye can be easily dissolved, however, in any buffer outside the range of pH 4 to 5. For the measurements presently to be described the dye was directly dissolved in the buffer used for the particular experiment.

The potential range of gallocyanine is rather close to that of methylene blue, a desirable property in view of the known inadequacies of the latter and the importance of the range it covers (1, 7). Moreover, such a duplication is desirable because of the fact that methylene blue, and the similar Lauth's violet and toluylene blue, are basic dyes, whereas gallocyanine at the pH range of biological importance acts as an acid dye. This dye may therefore be very useful in biology.

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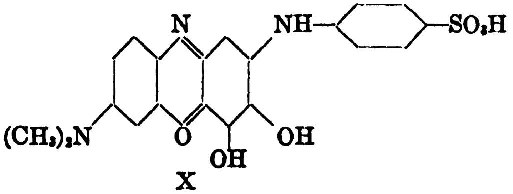
[3] See Schultz (4) No. 626, or (5) No. 883.

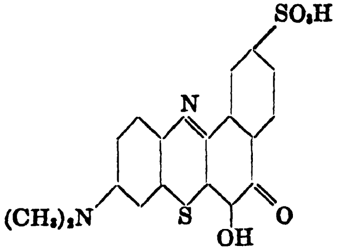
2. Gallophenine.[3]



This is a sulfonated oxazine. X stands for a monovalent acid residue.[4] It is easily soluble at any pH. It is usually blue, but turns green at very strong acid reaction and purple in a strong alkaline solution.[5]

The potential range of this dye is somewhat more negative than that of indigo disulfonate, approximating closely that of indigo monosulfonate. It is by far superior to indigo monosulfonate because of its high solubility. The solubility of indigo monosulfonate is so slight that Sullivan, Cohen, and Clark had to use approximate data concerning potentials. For this reason gallophenine may serve as a very desirable substitute for indigo monosulfonate.

3. Brilliant alizarin blue[6] (B. Heymann).



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[3] *Cf.* (5) No. 879.

[4] This formula has been written here as an *o*-quinoid structure. One could write it just as well in the form of a *p*-quinoid structure.

[5] This dye, in Schultz' Farbstofftabellen, is mentioned only in the index, not in the text.

[6] See Schultz (4) No. 667, or (5) No. 931.

This is a sulfonated thiazine. Its solubility in pure water is, though not high, entirely sufficient. When the dye is added to pure cold water, there is an undissolved residue; but when the filtrate is mixed with a buffer of pH around 7 it remains clear and behaves as a true homogeneous solution. Especially the titration curve, as described in the following, precisely fits the course expected for a truly dissolved and completely reversible dye, if the concentration of the dye is small enough. The filtered pure aqueous solution is the adequate form of a stock solution and should be mixed with the buffer solution desired immediately before use.

The dye has a potential range more negative than any of the indigo sulfonates and therefore represents a valuable extension of the series of really reversible redox indicators, though there may be cases where the peculiarity of this dye with regard to its solubility may cause difficulties.

The solution of the dye is blue-violet in water. At very low pH (approximately 1) it changes a little towards blue. The violet becomes more pronouncedly blue at pH 7, and remains so down to very alkaline ranges.

Methods Used for Titrations

The methods of tritration have been so carefully described by Clark that only little need be said in this respect. We wish, however, to add two items, one of perhaps only theoretical, the other of practical significance.

1. Use of Mercury Electrode in Presence of Hydrogen Gas.—When the oxidized form of the dye is reduced by a reductant such as sodium hydrosulfite, in many cases the mercury electrode can be used as an indifferent electrode even in an atmosphere of hydrogen gas. Mercury shows no inclination to work as a hydrogen electrode in the presence of hydrogen gas, and the potential of the reversible redox system can therefore be measured at the mercury electrode provided this potential is negative enough to reduce or perhaps eliminate by complex formation those traces of mercury ions which may be formed in the beginning of the experiment, and, on the other hand, is not too negative, the limit in this respect being set by the fact that even pure hydrosulfite shifts the potential of the mercury electrode no further than a poorly defined value 100 millivolts or so more positive than the hydrogen electrode at the same pH. The use of the mercury electrode is therefore restricted to a potential range, at pH 7, from about +0.1 to −0.2 or −0.25 volt, referred to the normal H_2 electrode. A great many

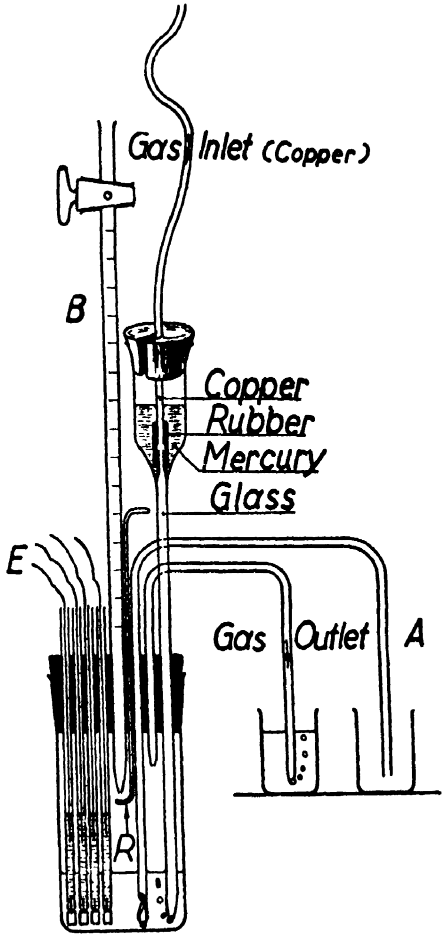

FIG. 1. Schematic drawing of the electrode vessel. E, electrodes; B, burette (notice the glass rod, R, next to the burette. It serves to catch the drop of fluid adhering to the tip of the pipette); A, agar lever, saturated with KCl (notice the stop-cock at its lower end). The whole vessel is held by a clamp which, by means of a motor, can bring about circular movements of the whole vessel around a perpendicular axis which lies outside the central axis of the vessel. The gas inlet is a flexible copper tubing allowing the electrode vessel to be moved freely.

of the preliminary measurements for orientation were made by this method.

2. Use of Colloidal Palladium for Titration.—Most of the measurements were performed in the following way. The electrode vessel (Fig. 1) was a cylindrical bottle, of such dimensions that the solution to be titrated (25–30 cc.) occupied a depth of only about 3 to 4 cm., to allow stirring. The bottle was closed by a rubber stopper through which were fixed:

A glass tube as a gas inlet. The gases were purified over heated copper, as described by Michaelis and Flexner (8), and conducted through copper tubing. As the description of the connection of the gas inlet in the paper just mentioned seems a little too brief, it may be given in more detail here. The copper tubing coming from the furnace is fixed as shown in Fig. 1. The opening is mounted with a short rubber tubing (Fig. 1). This is stripped over glass tubing and the glass calix is filled with mercury. A loosely fitting rubber stopper with a slit holds the copper tubing in place and prevents the mercury from splashing when the whole apparatus is being stirred.

A glass tube as gas outlet.

An agar tube saturated with KCl as bridge. The most suitable form, which at the same time leaves a minimum of contact surface for diffusion, was this. The glass lever (A, Fig. 1) is open at the end but carries a not too well ground stopper at the other end. The handle of the stopper should not have a larger diameter than the glass tube, so that it can be pushed through the hole of the rubber stopper of the electrode vessel. This glass stopper has on its upper end a hook and the glass tube contains inside a small thickened spot which serves as a support for the hook. The stopper is turned into the fitting position. The whole lever is filled with agar saturated with KCl, and the glass stopper fitted in, not too tightly, so as to leave a capillary agar connection. This stopper, after use, may be regenerated by keeping it under saturated KCl solution, without its being necessary to refill the whole tube each time.

A platinized platinum electrode, by which the pH of the solution after complete reduction is measured directly. On measuring the pH directly one need not rely on the pH as calculated from the composition of the buffer. One may work then even with buffers which are not standardized; *e. g.*, with phosphates of a concentration other than that of the standard solution. This is especially important when a dye has to be dissolved in pure water and a buffer is added later.

Three blank electrodes, say two of blank platinum and a gold-plated one.

A burette (B) calibrated in hundredths of a cc., the glass cock being located close to the upper end, above the zero point of the graduation.

It is very useful to have a glass rod (R) with small spoon-like hook. This rod should fit tightly, but be movable. It serves to catch an adhering drop of the oxidant from the tip of the burette during the titration.

In the beginning of the experiment 0.1 to 0.3 cc. of a solution of colloidal palladium according to Nacht-Pahl (1 gm. to a liter of water) is added and the reduction is carried out by means of a stream of hydrogen. After complete reduction the pH is measured with the platinized electrode. Now the gas lead is shifted to nitrogen, and the hydrogen is expelled from the solution and from the palladium. The potential of the platinized electrode is watched during this period. The removal of the hydrogen gas may safely be considered as complete for any practical purpose when the potential of the platinized electrode has become more positive by 80 to 100 millivolts. The crucial test for the complete removal of hydrogen is the observation that the very first drop of the oxidant added to the solution brings about not only an immediate coloration, but a coloration which persists indefinitely. A trace of hydrogen that may have remained will reduce the dye over again.

One may be surprized that the removal of hydrogen from palladium can be brought about so completely in a relatively short period of time (30 to 60 minutes). One may, however, take into consideration the fact that the total amount of palladium contained in the solution is a small fraction of 1 mg. and that the ultramicroscopic particles of palladium are brought to absorption equilibrium with the surrounding gas much more quickly than a macroscopic particle of the metal.

As this electrode vessel is attached to the gas inlet in an elastic, flexible manner, the stirring can be produced by shaking, and there is no need for a stirring rod with all its inconveniences. The stirring, after each addition of the content of the burette, may be done by hand, or better, by a shaking apparatus which executes circular movements of the vessel around a perpendicular axis. This arrangement allows one to read the potential either at rest or during the shaking and to study the influence of shaking. In well poised systems, there is a very small but distinct difference, noticeable even in the case of a hydrogen electrode with platinized platinum. Usually the potential is more negative by some tenths of a millivolt at rest than while being shaken. The potential at rest is to be considered as the desired value, because the thermodynamic theory on which the establishment of the potential is based does not take into account a mechanical movement of the electrode relative to the liquid in contact with it, which may interfere mechanically with the establishment of the electric double layer responsible for the potential difference. On the other hand without a thorough previous shaking there is no guarantee of a definite state of equilibrium throughout the whole system including the surface layer of the liquid immediately adjacent to the electrode.

The whole apparatus, consisting of the electrode vessel, a great part of the copper leads for the gases, the shaking apparatus, and the calomel electrodes, was kept in a compartment of constant temperature large enough to work in conveniently. The temperature was controlled by a toluene-mercury regulator, the heat being furnished from freely suspended heating wires of nichrome, the heat of which was blown off by an electric fan. The temperature of a bottle with water of the heat capacity of the electrode vessel was constant within much less than 0.1°, the constant temperatures being reached within 20 to 30 minutes. Almost all these experiments were made at 25°. Some experiments at 35° show that the temperature coefficients of the dye potentials are very small.

In most cases quinone was used as oxidant, sometimes also phenol indophenol or ferricyanide. The solution should be deaerated before pouring it into the burette. The air may be removed simply from the quinone solution by briefly boiling and immediately sucking it into the burette.

Result of Titration Experiments

1. Gallocyanine.—Fig. 2 shows one example of a titration experiment at constant pH. The dye is dissolved in a phosphate buffer, and quinone is used as oxidant. The dye is reduced by H_2 and palladium as described before, pH is measured with the platinized electrode, H_2 is expelled by purified N_2, and the titration is performed. Three electrodes, two of blank platinum and one of gold-plated platinum agreed, with respect to the potential, within a few tenths of a millivolt, except for the very beginning and the very end of the titration where the disagreement between different electrodes may be greater, but irrelevant for the shape of curve. The potential was established practically instantaneously after stirring, and was constant for any length of time. The difference between the potential reading during the stirring or in the state of rest was never sufficiently large to appear in the plotting on the scale in Fig. 2. The values at rest were used for the diagram. The drawn out curve is the one theoretically calculated according

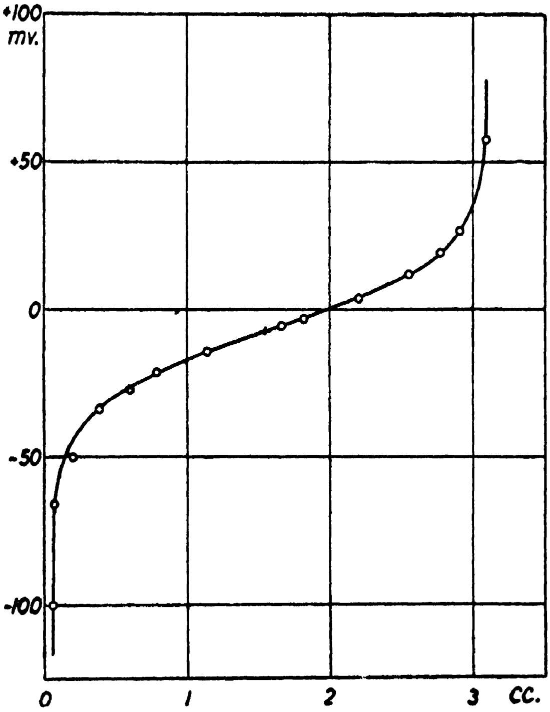

FIG. 2. Gallocyanine, reduced and then titrated with quinone, at 25.0°. pH = 7.390, phosphate buffer; abscissas denote cc. of quinone solution, ordinates, potential referred to the normal hydrogen electrode, in millivolts.

to the principles set forth by Clark. From this curve E_0', or the potential of the dye in the half reduced state referred to the normal hydrogen electrode, is best evaluated graphically. The assumption underlying the calculation of pH is that the pH of standard acetate is 4.62. From this assumption the potential of the calomel electrode used referred to the normal hydrogen electrode, was calculated, and this standardization of the calomel electrode was frequently repeated during the course of the series of individual titration experiments. Only one titration experiment is shown in detail, but all the others were used similarly to calculate the E_0' at various pH graphically shown in Fig. 3. Borate buffer could not be used because the reduced form of the dye has two phenolic hydroxyl groups. Experiments with borate as buffer gave a curve deviating markedly from that expected for an undisturbed system of reversible dye, the curve being much too flat in the beginning. When, by fitting best values to such an unsatisfactory curve, a trial calculation of E_0' was made, a value quite outside that of the theo-

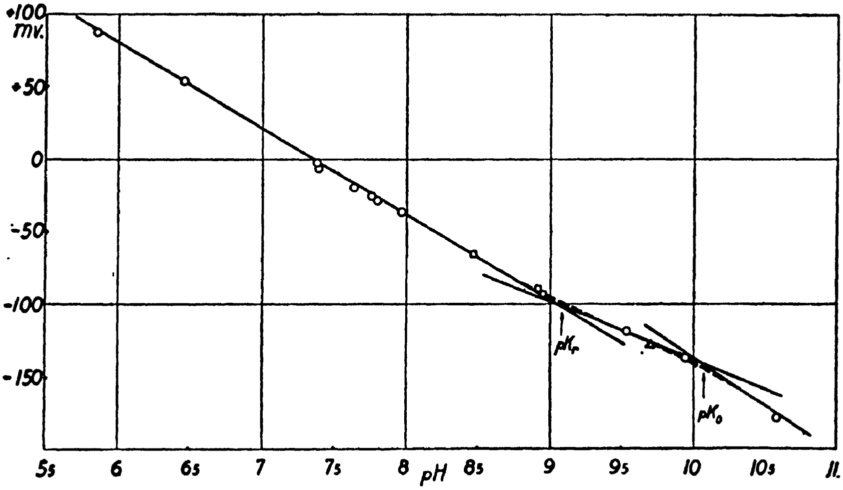

FIG. 3. Potential of gallocyanine in the half reduced state at varied pH, at 25.0°.

The abscissas denote pH; ordinates, potential, referred to the normal hydrogen electrode, in millivolts. Buffers, ○ phosphate, □ veronal, △ glycocoll. The curve as interpolated from the experiments is dotted. The tangents of 0.06 slope respectively 0.03 slope are drawn out. pK_r, pK_o, dissociation constants of the reduced form and the oxidized form respectively.

retical curve was found. None of the following buffers showed this specific effect: Sörensen's phosphate buffers, mixtures of secondary phosphate and NaOH, acetate buffer, veronal buffer (recently recommended (6)), and glycocoll buffer.

The diagram, Fig. 3, shows that the slope of 0.0591 volt shift per unit of pH holds between pH about 6 to 9. There is a deviation from this slope towards the acid side from 5.5. This part of the curve could not be investigated in more detail because at pH < 5.5

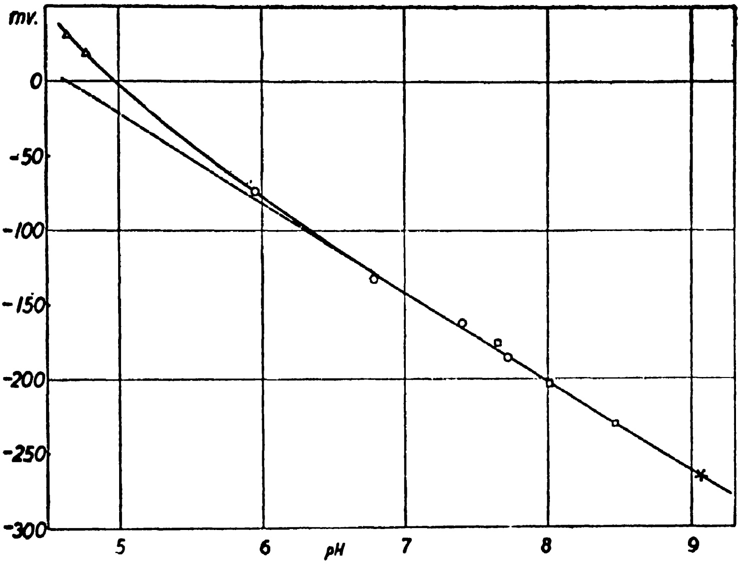

FIG. 4. Potential of gallophenine in the half reduced state, at varied pH, and at 25.0°. The abscissas denote pH; ordinates, potentials referred to the normal hydrogen electrode, in millivolts. Buffers, △ acetate, ○ phosphate, □ veronal, * glycocoll.

we approach that range of pH in which the solubility of the dyes becomes too slight to allow accurate titration experiments, and the figures obtained in this range are omitted in the diagram. A second deviation is encountered between pH 9 and 10. This range of deviation of the slope coincides with the range of color change of dye from blue to red-violet, indicating that it is due to the effect of an acid or basic dissociation constant of the dye, as Clark has shown in many instances. On applying his principles one may calculate graphically a dissociation constant for the oxidized form of the dye, pK_o, and another for the reduced form, pK_r. This is performed by drawing the best fitting tangent with the slope of $\frac{0.0591}{2}$ volt per unit of pH. The two points of intersection of such tangents give the two dissociation constants at the abscissa. The first of these, $pK_r = 9.1$ owing to the fact that the curve is flattened at this region, according to the theory belongs to the reduced form, the second, $pK_o = 10.1$, to the oxidized form, because the curve becomes steeper.

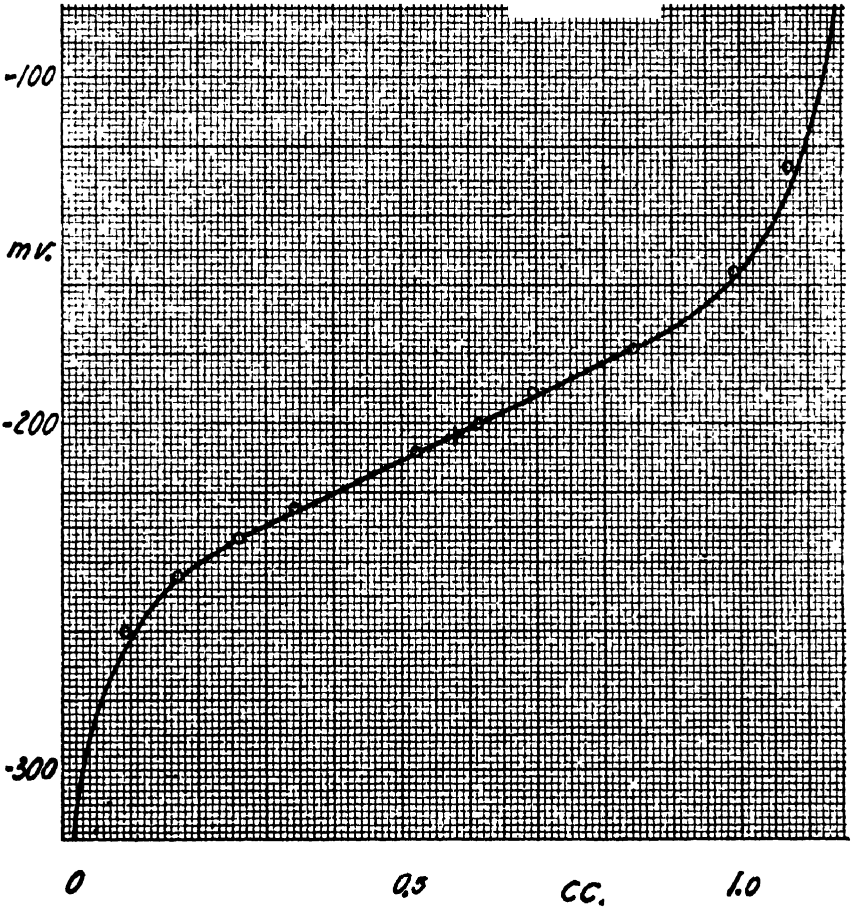

FIG. 5. Titration of reduced brilliant alizarin blue with quinone. pH = 5.918. Abscissas denote cc. of quinone solution, ordinates, potential in millivolts referred to the normal hydrogen electrode.

2. Gallophenine.—The data for E_0' at various pH are plotted in Fig. 4. The curve has a slope of 0.0591 volt per pH unit, and the observed points fit into this straight line throughout the whole range of pH investigated, except for pH <6, where a dissociation constant of the dye obviously interferes.

3. Brilliant Alizarin Blue G.—Here one individual titration experiment is shown in detail (Fig. 5), and the result of all experiments is plotted in Fig. 6. The slope is regular between at least pH 5.5 and 7.8 within the limits of error, then flattens and regains the normal slope from about 8.6 into the most alkaline range investigated. The intermediary part of the curve, around pH 9, could be studied only by means of the veronal buffer. The points around pH 7.5, where the phosphate and the veronal buffers overlap, were studied with either buffer, and a perfect agreement was obtained.

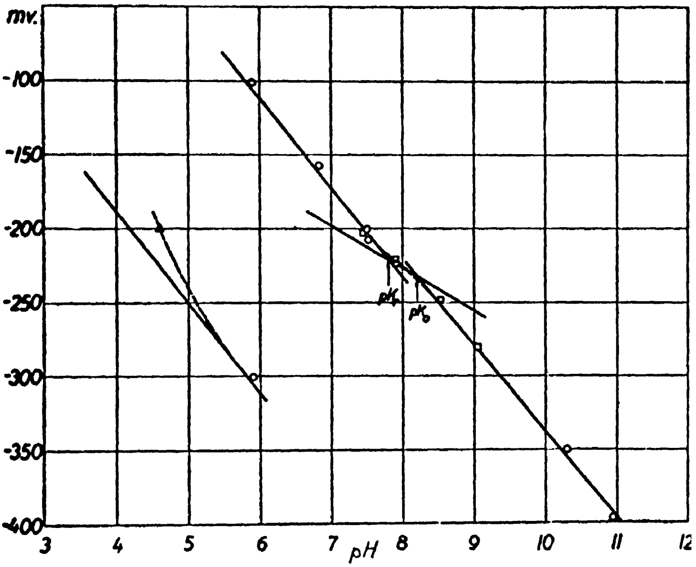

FIG. 6. Potential of brilliant alizarin blue, in the half reduced state at varied pH, at 25.0°. The abscissas denote pH, ordinates, potential in millivolts, referred to the normal hydrogen electrode. The left-hand curve is an extension of the right-hand curve to the left, with respectively shifted ordinates, to save space. The drawn out lines are the tangents with the slope 0.0591 respectively 0.02905. pK_r, pK_o are dissociation constants of the reduced form and the oxidized form respectively. Buffers, ○ phosphate, □ veronal, △ acetate.

On applying the graphic construction mentioned above one obtains as a dissociation constant for the reduced dye $pK_r = 7.8$, and for the oxidized dye $pK_o = 8.2$. The deviation from the straight line throughout the pH range 6 to 11 is small indeed. The straight line between pH 6 and 8, and the one between 9 and 11, are only 0.1 unit of pH apart. Yet we do not believe this separation to be within the errors of the experiments; even when we allow for errors within reasonable limits it is impossible to connect all observed points from pH 6 to 11 by one straight line of the slope 0.0591 per unit of pH.

TABLE I.

Potentials of the Half Reduced Dyes Referred to the Normal Hydrogen Electrode at 25°.

pH	Brilliant alizarin blue.	Gallophenine.	Indigo disulfonate.	Methylene blue.	Gallocyanine.
5	−0.040	−0.003	−0.10	+0.101	
5.25	−0.062	−0.023			
5.5	−0.080	−0.042			+0.110
5.75	−0.097	−0.060			+0.095
6	−0.112	−0.077	−0.069	+0.047	+0.080
6.25	−0.127	−0.094			+0.065
6.5	−0.143	−0.110			+0.051
6.75	−0.157	−0.127			+0.46
7	−0.173	−0.142	−0.125	+0.011	+0.021
7.25	−0.188	−0.157			+0.007
7.5	−0.203	−0.172			−0.008
7.75	−0.216	−0.187			−0.023
8	−0.226	−0.202	−0.167	−0.020	−0.037
8.25	−0.237	−0.217			−0.042
8.5	−0.250	−0.232			−0.067
8.75	−0.265	−0.247			−0.082
9	−0.279	−0.262	−0.199	−0.050	−0.095
9.25	−0.293	−0.276			−0.107
9.50	−0.309	−0.291			−0.118
9.75	−0.323				−0.128
10	−0.337				−0.140

The values for indigo disulfonate and methylene blue are shown for comparison and have been taken from the measurements of Clark and his associates, at 30°.

In using these dyes as redox indicators the following suggestions with regard to solubility may be made. Gallocyanine is best dissolved directly in the buffer to be used. Gallophenine is so easily soluble under any condition that no special precautions are necessary. Brilliant alizarin blue is best dissolved in pure water, filtered, and this filtrate may be used as a stock solution and added to the buffer to be used. If prepared in this way the solution of the dye in the buffer behaves as a true homogeneous, non-colloidal solution, as can be inferred from the titration curves.

All these experiments were made at 25° ± 0.05°.

A few experiments were made at 35°, and the temperature coefficient turned out to be very small. Not enough data are available as yet to give accurate figures. Table I gives the E_0'; *i.e.*, the potential of the half reduced dye, at 25° and at various pH, and some comparative data at 30° for the dyes of Clark's series.

SUMMARY

Three new redox indicators are characterized, and their potentials summarized in Table I.

Technical details for general measurement of reversible redox systems and their titration are described.

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RESPIRATION OF MAMMALIAN ERYTHROCYTES

BY L. MICHAELIS AND K. SALOMON

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Accepted for publication, May 5, 1930)

1. Respiration of Erythrocytes Induced by a Respiratory Supplement Prepared from Various Tissues

Lavoisier's fundamental interpretation of respiration as oxidation of organic compounds by free oxygen is valid even at the present time without restriction. His opinion was that this oxidation takes place in the blood. This hypothesis had to be given up, the respiration really taking place in the tissues. Red corpuscles, at least the non-nucleated corpuscles of mammals, do not participate in the reduction of oxygen to any appreciable extent.

Recently, however, Harrop and Barron (1, 2) showed that non-nucleated erythrocytes do respire in the presence of methylene blue and some other related dye stuffs, which can be designated, therefore, as catalysts for respiration. All of these artificial catalysts are alien to the animal body, and no component of any tissue has ever been shown to exhibit the same effect as methylene blue on erythrocytes. Never has a respiration of non-nucleated erythrocytes in any way comparable in extent to that of other tissues been observed prior to the experiments of Harrop and Barron.

It will be shown in this paper that a substance can be extracted by water from various organs which induces non-nucleated erythrocytes to respire just as methylene blue does. This substance will be shown not to be identical with Warburg's (3) respiration enzyme because it is not sensitive to carbon monoxide.

Methylene blue was used by Thunberg (4) as an oxidant instead of oxygen, for something that may be reckoned as respiration only when the meaning of this concept is considerably widened. In his experiments, methylene blue is a hydrogen acceptor, instead of oxygen, which is absent throughout the experiment. Different from these experiments is the use of methylene blue in the presence of oxygen as catalyst for the consumption of oxygen by cells. Meyerhof (5) was the first to show that methylene blue sometimes acts as a catalyst for respiration in air. He observed this property of methylene blue, however, only in cell extracts the respiratory faculty of which had been artificially damaged, such as in heated extract of yeast previously treated with acetone. The respiration of the yeast cells themselves could not be improved by methylene blue under normal conditions. Not even the oxygen consumption of the extract of acetone yeast was altered by methylene blue unless the spontaneous oxygen consumption of such an extract was damaged by heating. Only a damaged respiration could be improved by methylene blue, this dye acting as a substitute for the destroyed respiration enzyme.

Later on it was shown by Harrop and Barron (2a), that certain cells which by nature exhibit a very low respiration, or none at all under natural conditions, can be stimulated to an intense respiration by methylene blue. This has been shown for erythrocytes and for unfertilized echinoderm eggs. The effect of methylene blue, according to Barron, is shown also for other dyes reversibly oxidizable and reducible. The magnitude of this effect appeared to depend on the potential range of the dye, methylene blue showing the optimum effect, whereas dyes with a more positive or a more negative range of potential have smaller effects or even no effect at all. As for the interpretation of this observation, we may refer to Barron's paper.

The reaction of a physiologist's mind to this observation may be, and has been, different. One point of view is that this effect of methylene blue is an accidental property of the dye, that no substance analogous to the dye exists in the living organisms, as seemingly shown by the fact that erythrocytes really do not respire. Another point of view is the hypothesis that methylene blue might be a model for some unknown substance in the organisms, and that this substance may play a part in respiration, which is not obvious as yet. This hypothesis is supported by the observation presented in this paper, showing that extracts of various organs induce erythrocytes to respire just as methylene blue does. The best effect was obtained with liver extract of various animals, and rat's liver seems to be the most suitable tissue for the extraction of the active substance, to which we will refer in what follows under the term *respiratory supplement.* Then follows kidney, spleen, testicle, lymph-node, whereas brain, muscle, and blood serum showed no effect or at most a very slight effect. Ox organs showed similar effects, the action often being somewhat less intense, but the present method is not yet adapted for a quantitative extraction of the supplement or for the estimation of its original concentration in the living organ.

The respiration of blood corpuscles induced by methylene blue or by organ extract is not influenced at all by the presence of carbon monoxide. Rabbit's erythrocytes, in the presence of methylene blue or rat's liver extract, respire at the same rate in air or in a mixture of pure carbon monoxide containing a small amount of oxygen, varied from 1 to 2 per cent in different experiments. Such a gas mixture inhibited the rate of respiration of yeast or of nucleated erythrocytes of fowl by approximately 60 to 80 per cent depending on the conditions, in accordance with Warburg's observations. This difference in the behavior toward CO proves that the supplement is not identical with Warburg's respiration enzyme.

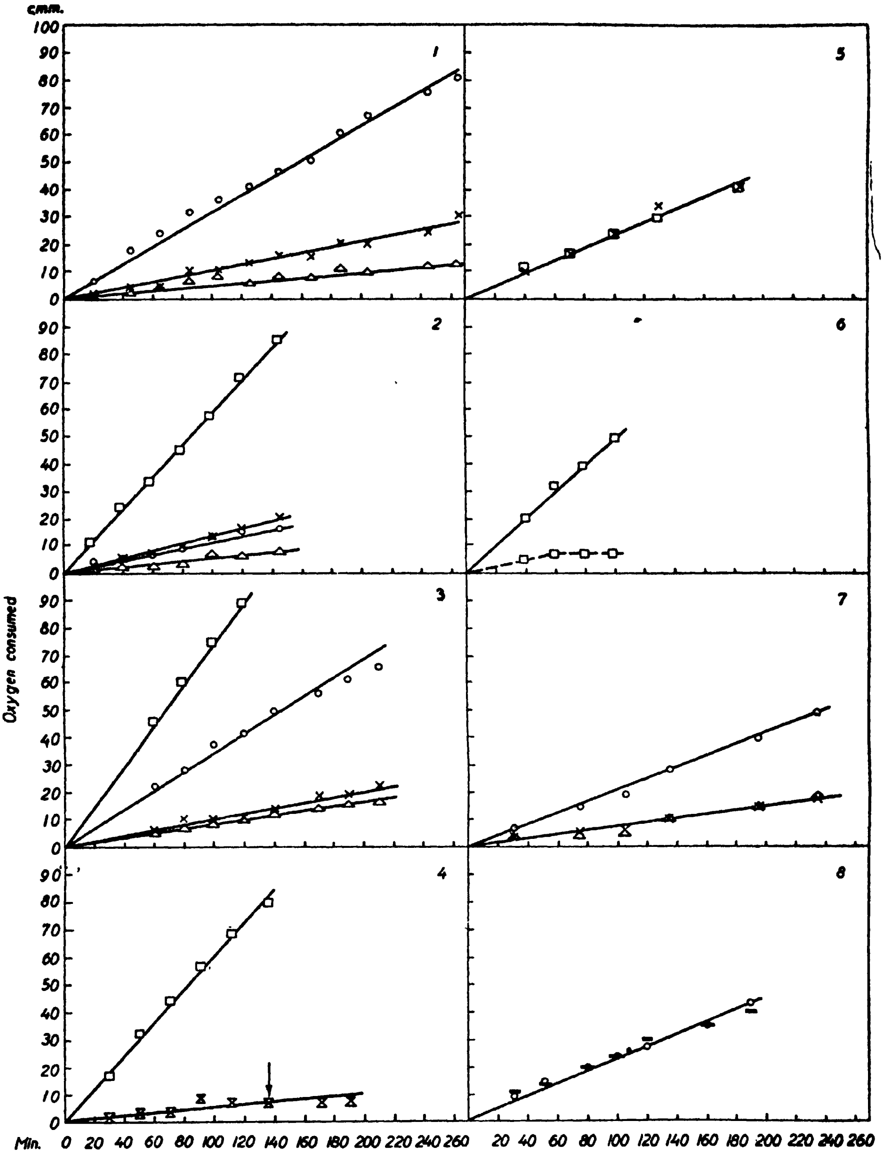
The end products of this respiration and the respiratory quotient will be investigated in a subsequent paper.

In varying the temperature from 23° to 37°, the rate of respiration of blood corpuscles induced by methylene blue showed practically no difference, whereas the respiration induced by organ extracts was two to three times as fast at 37° as compared with 23°. In general, the organ extracts, at 23°C., are less active than methylene blue in its optimum concentration (0.0006 molar), but are considerably stronger than methylene blue at 37°.

As a preliminary statement it may be added that the effect of methylene blue, though very strong even in the dark, is considerably increased by illumination. The effect of organ extracts is the same in the dark or when illuminated by a 150 watt bulb at a short distance.

Methylene blue had no influence upon that slight oxygen consumption exhibited by organ extracts alone in the concentrations used.

The influence of narcotics has been studied only for phenyl urethane as yet. This showed no influence at all upon the effect of methylene blue or of the supplement.



Figs. 1 to 8 at 23°C.

Common designations for all curves:

○ Experiments with blood, sugar and organ extract.

× Control with blood and sugar.

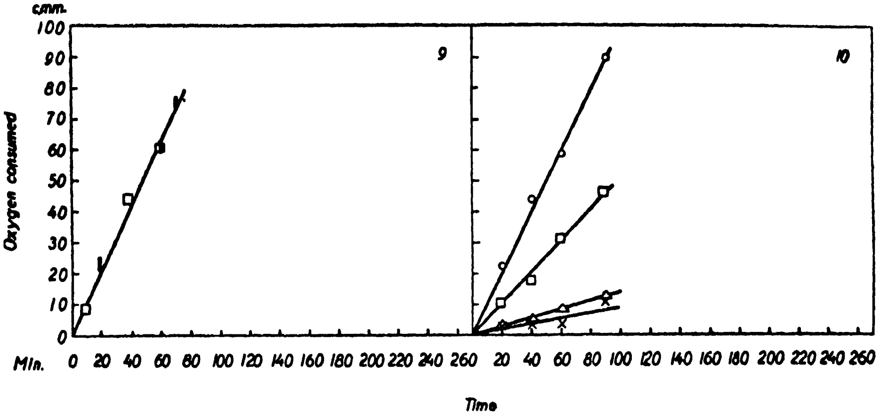
△ Control with organ extract and sugar, without blood corpuscles.

□ Experiment with blood, sugar and methylene blue.

⧖ Control with blood plus methylene blue, without sugar.

- - - - Experiments with hemolyzed blood (Fig. 6).

▬ Experiments with blood, sugar, organ extract in carbon monoxide containing 1.4 per cent oxygen (Fig 8).



Figs. 9 and 10 at 37°C.

Common designations for all curves:—*continued.*

|| Experiments with blood, sugar, methylene blue in carbon monoxide with 1.4 per cent oxygen.

Fig. 1. Effect of rat liver extract at 23°C. The respiration in presence of rat liver extract is far greater than the sum of the respirations in all controls.

Fig. 2. Effect of methylene blue (□) compared with that of rat muscle extract (○), at 23°C., in the presence of sugar. Muscle extracts show no effect in this case (a very slight effect in other cases, especially at 37°).

Fig. 3. Comparison of methylene blue (□) and rat kidney extract (○) at 23°C. At this temperature organ extracts as a rule act less intensely than methylene blue.

Fig. 4. Shows that methylene blue increases the respiration only in presence of glucose (□) whereas it has no influence in absence of sugar (X). When sugar is added after the methylene blue has been in contact with the blood corpuscles for 2 hours, subsequent addition of sugar induces no respiration. The arrow shows the time of addition of glucose.

Fig. 5. For chicken erythrocytes respiration is not influenced by the addition of methylene blue even in presence of sugar. Temperature 23°C. The spontaneous respiration of these nucleated erythrocytes in sugar is rather high alone even at 23°C., and cannot be increased by methylene blue.

Fig. 6. The effect of methylene blue is present only for intact erythrocytes but not after hemolysis.

Fig. 7. Effect of rat spleen on the respiration of rabbit erythrocytes in presence of sugar. 23°C. The effect is somewhat smaller than with liver.

Fig. 8. The respiration of rabbit erythrocytes induced by rat liver extract is equal in air (○) or in carbon monoxide containing 1.4 per cent oxygen (=).

Fig. 9. The respiration of rabbit erythrocytes in the presence of glucose induced by methylene blue, is equal in air (□) or in carbon monoxide containing 1.4 per cent oxygen.

Fig. 10. Effect of rat liver extract (○) or of methylene blue (□) on the respiration of rabbit erythrocytes with glucose at 37°. At this temperature the effect of organ extract is greater than that of methylene blue.

On discussing the chemical nature of this respiratory supplement it might be a suggestive assumption that glutathione or another sulfhydril body is the responsible substance of the tissue extracts, and to link these observations with Hopkins' interpretation of glutathione as a catalyst for respiration, owing to its property of being readily reduced and reoxidized. Meyerhof also showed such an effect for thioglycolic acid and thiolactic acid, though it was only exhibited on structure-free cell extracts. Such an idea was even more suggestive as just those tissues which contain no, or very little, glutathione, namely, blood serum and muscle tissue, show no, or a very small, effect on the respiration of blood corpuscles. Our experiments, however, do not as yet support this hypothesis. Reduced glutathione, or cysteine, added to blood corpuscles in Ringer solution plus glucose, consumed just as much oxygen as was required for the oxidation to the disulfide. When added in such small amounts that this oxygen consumption could be practically neglected, they had no effect whatsoever. When glutathione in the oxidized state or cysteine was added, no effect could be seen at all, with or without addition of an iron salt.

A second possibility was that the respiratory supplement might be identical with Harden and Young's (7) coenzyme. Meyerhof showed that the coenzyme enhances not only fermentation but also oxidation. However, this idea has to be abandoned too, because muscle contains by far the greatest amount of the coenzyme among all tissues, according to Meyerhof, whereas our effect is exhibited by muscle extract almost to the least degree among all tissue extracts. The fact that muscle extract contains so little supplement is also contradictory to the suggestion that Keilin's cytochrome be identical with the supplement.

On searching for another explanation, one may think of the fact that it is just cells without a nucleus (erythrocytes) or cells with a nucleus in an obviously resting state (unfertilized eggs) which can be induced to respire by methylene blue. Perhaps the respiratory supplements of organ extracts, analogous to methylene blue, is some substance derived from the cell nucleus. In fact, tissues with no nucleus (serum) or with very few nuclei per unit of mass (muscle, brain), have almost no effect. This idea is, at the present time, a mere suggestion.

The respiration of erythrocytes as described before, either under the influence of methylene blue or of the supplement, is shown only by intact erythrocytes. Erythrocytes hemolyzed by distilled water, or by repeated freezing, show no respiration, or when the hemolysis is incomplete there is a diminution of the respiration according to the extent of hemolysis.

The respiration of chicken erythrocytes is not influenced by methylene blue.

There is in the literature on respiration one observation which may have a connection with ours. Battelli and Stern (8) showed that what they call the accessory respiration of a tissue or a tissue extract (such as liver or kidney) is noticeably, though not strongly, increased by addition of washed blood corpuscles. This observation, however, is made under rather different experimental conditions and differs from ours by the fact that the effect described by Battelli and Stern is the same whether the blood corpuscles are added in the intact or in the hemolyzed state, whereas our effect is strictly confined to the intact state of the erythrocytes.

2. *The Influence of the Medium upon the Action of the Respiratory Supplement*

The medium in which most of the experiments were performed is Ringer solution with 0.18 per cent of glucose. In sugar-free Ringer solution methylene blue shows no effect, at least when the erythrocytes are used in not too fresh a condition, where the natural sugar content of the blood corpuscles may be supposed to be not yet consumed. But even in very fresh condition, when perhaps not all of the natural sugar content of the blood corpuscles may have been destroyed, the respiration of blood corpuscles under the influence of methylene blue is very much smaller in sugar-free Ringer solution than it is in a medium containing sugar. When sugar-free blood corpuscles suspended in Ringer solution containing glucose are mixed with methylene blue, a strong oxygen consumption starts immediately and goes on at a constant rate over several hours; indeed over a period of time such an experiment of this sort can reasonably be extended, 5 to 6 hours at 23°, or 2 to 3 hours at 37°. Methylene blue does not harm the blood corpuscles within this period. When, however, methylene blue is added to a suspension of blood corpuscles in a sugar-free medium, the dye seems to exhibit a damaging influence to the blood corpuscles. For not only is the respiration missing under this condition, but also the respiration is not established on adding glucose after an interval of 1 to 2 hours. One may think of the following interpretation: If there is no sugar the oxidation catalysed by methylene blue is directed toward some other oxidizable substance in the cell which is important for its physiological structure. This substance is protected from oxidation in the presence of sugar.

Experiments of the same kind but with organ extracts instead of methylene blue did not lead to any interpretable result because such extracts cannot be obtained free from sugar or glycogen.

The effect of methylene blue can be shown to the same extent within the limits of error by substituting fructose or saccharose for glucose. In contrast, there was no respiration with mannite. Ringer solution with mannite behaves like sugar-free Ringer solution.

On attempting to replace Ringer solution in the methylene blue experiments by various isotonic salt solutions, the following results were obtained. The oxygen consumption in 0.9 per cent NaCl solutions was somewhat smaller than in Ringer solution. Omitting only $CaCl_2$ from the Ringer solution the respiration was a little smaller too, when compared with the control with complete Ringer solution. By substituting for the very small amount of $NaHCO_3$ of the Ringer solution an equivalent amount of alkali phosphate, no distinct difference could be found, no matter whether this small amount of phosphate was added in the form of primary or secondary phosphate or of a mixture of both. Phosphates at a higher concentration, however, have a decidedly inhibiting effect.

When the blood corpuscles are hemolyzed by dissolving them in distilled water and salt solution is added, such as to give the final mixture the composition of a Ringer solution, no respiration takes place at all either with or without methylene blue, either in the presence or in the absence of sugar, provided the hemolysis is complete. With incomplete hemolysis the result may be intermediary. Hence, the respiration faculty of red blood corpuscles is linked with what is called the structure of the cell just as much as is the glycolytic faculty. None the less, phenyl urethane has no influence on the respiration induced by liver extract.

3. *Preliminary Attempts to Characterize or Isolate the Catalyst Supplement*

As yet we have not succeeded in isolating the efficient substance from the tissue extract or in separating by any means an active fraction of the extract from an inactive one. The following attempts were made:

a) Heating the extract at 80° destroys the active substance, at least the filtrate of the heated extract is without any effect. When, however the boiled turbid suspension as a whole was used the experiments were obscured by the fact that boiled extracts containing the coagulated particles show a very considerable oxygen consumption themselves which overshadows any oxygen consumption which they might induce.

b) When rat liver is ground in a mortar and extracted with acetone, neither the acetone-insoluble residue nor the residue of the acetone extract showed any effect comparable to that of the fresh tissue extract.

Exposure to light does not influence the effect of tissue extract, no matter whether the respiration takes place in air or in a mixture of 98 per cent CO + 2 per cent O_2. On the other hand, there is a remarkable effect of illumination upon the action of methylene blue. The effect of methylene blue, though it is very marked even in the dark, is strongly increased in the light. This preliminary observation will be elaborated in a subsequent paper.

4. *Experimental Part*

The oxygen consumption was measured by Warburg's micro-respiration apparatus with Haldane micro-manometer. The volume of the vessels was about 25 cc., and 7 per cent KOH was used as absorbent for CO_2. The temperature was in the majority of the experiments 23°C., in other experiments 37°, and constant within ±.01°. As a rule, rabbit erythrocytes were used, prepared by repeatedly washing citrated blood with the wanted salt solution, and suspending in a volume of the liquid equal to the original volume of the blood sample. In other experiments human erythrocytes were used with the same result. As a rule, fresh blood corpuscles were used. For especial cases, namely, when a strict absence of any trace of sugar was wanted, the corpuscles were used after 24 hours' standing in the ice box. No noticeable difference could be found, as a rule, whether the corpuscles were fresh or 1 day old, disregarding that very slight effect of a residue of glucose which is supposed to be left in fresh corpuscles.

The organ extracts were prepared by grinding the fresh organ in the mortar and gradually adding Ringer solution (or another medium), ten times the weight of the organ. This emulsion was filtered through ordinary filter paper and the turbid liquid free from coarse particles was used. These extracts were always used in fresh condition.

A selection of the experiments is reproduced in the following diagrams. The time "0" in these diagrams, plotted on the abscissa, is usually 20 to 30 minutes after closing the stop-cock of the manometer, because the very first stage sometimes shows irregularities and the linear course of the change of the manometer reading with time is often reached only after this period. From here the linear course is maintained over several hours. The ordinates give the oxygen consumption in cubic millimeters.

Each experimental mixture is composed as follows:

a) 2 cc. of a suspension of blood corpuscles in Ringer solution (containing all corpuscles of 2 cc. pure blood); or, in control experiment, without blood, the equal volume of pure Ringer solution instead.

b) 2 cc. organ extract, or the same volume of pure Ringer solution instead.

c) In the experiments with methylene blue instead of *b*), methylene blue is added to a definite concentration of 0.0006 molarity. (The variation of this concentration has a relatively small effect, down to about a tenfold dilution of the above concentration.)

d) In all experiments containing sugar the concentration is 0.18 per cent in the final mixture.

e) 7 per cent KOH was used as absorbent for CO_2.

SUMMARY

Non-nucleated mammalian erythrocytes do not respire even in the presence of sugar, but they do respire after addition of a small amount of methylene blue.

It is shown in this paper that aqueous extracts of various organs, especially liver, act in the same way as methylene blue. The respiration of erythrocytes induced by an organ extract is not altered in the presence of carbon monoxide.

The content of this respiratory supplement in extracts of organs varies according to the organ: liver and kidney show the best effect; muscle, brain, and blood serum the least.

With hemolyzed erythrocytes no respiration can be induced either by methylene blue or by organ extracts.

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SUSCEPTIBILITY OF RABBITS TO INFECTION BY THE INHALATION OF TYPE II PNEUMOCOCCI

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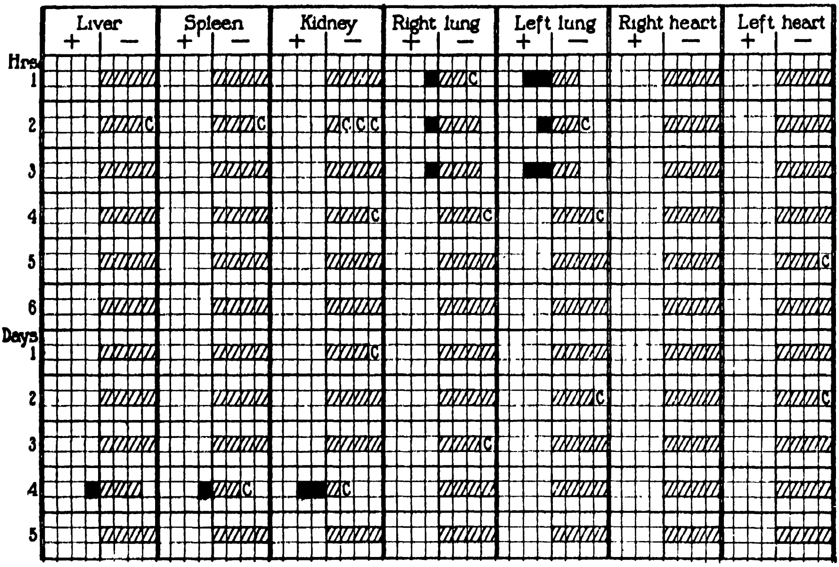
In preceding papers (1) it has been shown that following inhalation, virulent Type I pneumococci generally disappear from the lungs of rabbits within a few hours, only to reappear later in certain instances in which a general septicemia occurs. It has further been shown that some animals may recover from a Type I pneumococcus septicemia. Rabbits are, nevertheless, very susceptible to infection by Type I pneumococci. In order to determine how they would react to less virulent strains of pneumococci, Type II pneumococcus was selected. Three different strains of Type II were used. One strain was converted into the "R" form, by repeated transfers in Type II antipneumococcus serum. The changes from the "S" to the "R" form are accompanied by loss of capsule, type specificity, and virulence for mice. This strain is designated Pneumococcus IIR and is avirulent for rabbits. The original Pneumococcus Type II strain is designated Pneumococcus Type II (SAv). An intraperitoneal injection of 0.1 cc. of a broth culture of this stock strain is necessary to kill rabbits. It was rendered rabbit virulent by rapid passage. This virulent strain, called Pneumococcus II (Sv), regularly kills rabbits following intraperitoneal injections of 0.000,01 cc.

In the present paper are reported (1) the distribution of pneumococci in the organs of rabbits following exposures to repeated inhalations of the above mentioned strains, (2) the occurrence of positive blood cultures, and (3) the mortality of the rabbits exposed to each of the above mentioned strains.

Method

The rabbits were placed in a large spray chamber similar to that already described (2) and exposed to a spray of pneumococci. 150 cc. of an 18 hour broth culture was used for each spraying. The animals were exposed at 10 day intervals. Before each spraying, a sample of blood was obtained by bleeding the animal from the ear vein. At daily intervals after the first spraying, bood cultures were taken from a few of the rabbits. In the case of the animals which were killed and autopsied, broth cultures were made from both sides of the heart, and small pieces of the periphery of both lungs, kidney, liver, and spleen were placed directly in tubes of broth. Heart's blood cultures alone were made from the rabbits which died. All cultures were plated on blood agar for further identification. The animals that were killed were first chloroformed, immersed in a solution of lysol, and autopsied with sterile instruments.



TEXT-FIG. 1. Distribution of IIR pneumococci in organs following spraying. The black squares indicate organs in which cultures showed pneumococci to be present, the cross-hatched squares represent organs in which cultures remained sterile. "c" = contamination.

Distribution of Pneumococci IIR in the Organs

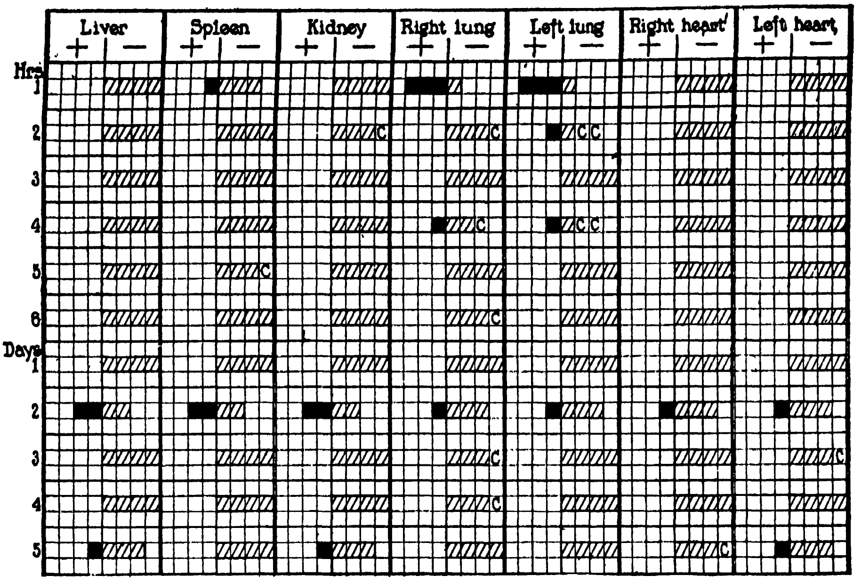
In Text-fig. 1 is shown the distribution of IIR pneumococci in the organs of 44 sprayed rabbits which were killed at intervals after spraying. One to 6 hours after spraying pneumococci were only recovered from the periphery of the lungs in less than half of the animals sacrificed within the first 3 hours. Pneumococci were never recovered in this group from the liver, spleen or kidney. The heart's blood cultures from these animals remained sterile. Of the remaining 20 rabbits killed from 1 to 5 days following exposure, pneumococci were only recovered from 2 rabbits killed on the 4th day. In one of these rabbits pneumococci were recovered from the liver, kidney and spleen and in another only from the kidney. This experiment shows how readily rabbits destroy the non-virulent R-forms of pneumococcus. These organisms were never recovered from the blood, rapidly disappeared from the lungs, and were rarely recovered from other viscera.

Daily blood cultures were taken from 7 rabbits following their first exposure to a spray of Pneumococcus IIR. During the 10 days period of observation in no instance were pneumococci recovered from the blood.

In all, 17 rabbits were exposed to from 3 to 10 inhalations of avirulent Type IIR pneumococci. Eight rabbits completed the course of 10 exposures. No rabbit became infected.

Distribution of Pneumococci Type II (SAv) in the Organs

In Text-fig. 2 is shown the distribution of the slightly virulent Pneumococci Type II (SAv) in the organs of 44 sprayed rabbits which were killed at intervals after spraying. It will be seen that pneumococci were recovered from the lungs of 3 of the 4 rabbits killed in 1 hour and that although the heart's blood cultures remained sterile, in one instance pneumococci were demonstrated in the culture from the spleen. After the 1st hour, however, the pneumococci were only recovered from the lungs in 3 instances. Of the remaining 20 rabbits killed from 1 to 5 days following exposure, 1, or 5 per cent, showed a general pneumococcus septicemia on the 2nd day. Although in the other rabbits pneumococci were not recovered from the lungs, in 2 instances the liver and kidney contained organisms and the spleen was positive in 1 rabbit. From the left heart's blood of one of the rabbits killed on the 5th day a positive culture was obtained but the culture of the right heart and spleen of this animal remained sterile. This experiment shows that a few rabbits may develop a generalised septicemia following inhalation of the slightly virulent Pneumococcus Type II (SAv).



TEXT-FIG. 2. Distribution of Type II (SAv) pneumococci in organs following spraying. The black squares indicate organs in which cultures showed pneumococci to be present; the cross-hatched squares represent organs in which cultures remained sterile. "c" = contamination.

TABLE I

Days on Which Positive Blood Cultures Were Obtained Following a Single Exposure to Inhalation of Pneumococci Type II (SAv)

Rabbit No.	Days										Course
	1	2	3	4	5	6	7	8	9	10	
1	—	—	—	—	c	—	—	—	—	—	Survived
2	c	—	—	—	c	c	—	—	+	+	"
3	—	—	—	—	—	—	—	—	+	—	"
4	+	+	—	—	—						Died 5th day. Blood sterile
5	—	+	—	—	—	—	—	—	—	—	Survived
6	—	—	—	—	—	—	—	—	—	—	"
7	—	—	—	—	—	—	—	—	—	—	"

+ indicates a positive culture of pneumococcus; — = a sterile culture; and "c" = a contaminated culture.

Blood Cultures

Daily blood cultures were taken from 7 rabbits following their first exposure to a spray of Pneumococcus II. From Table I it is seen that positive blood cultures were obtained from 4 of these rabbits. In 2 instances the blood was positive on but one occasion. In the other 2 rabbits the blood cultures were positive on 2 successive days. One of these rabbits survived, the other rabbit died. As the latter rabbit's blood had been sterile for 3 days and as the postmortem blood culture remained sterile, it is evident that death was not due to pneumococcus septicemia.

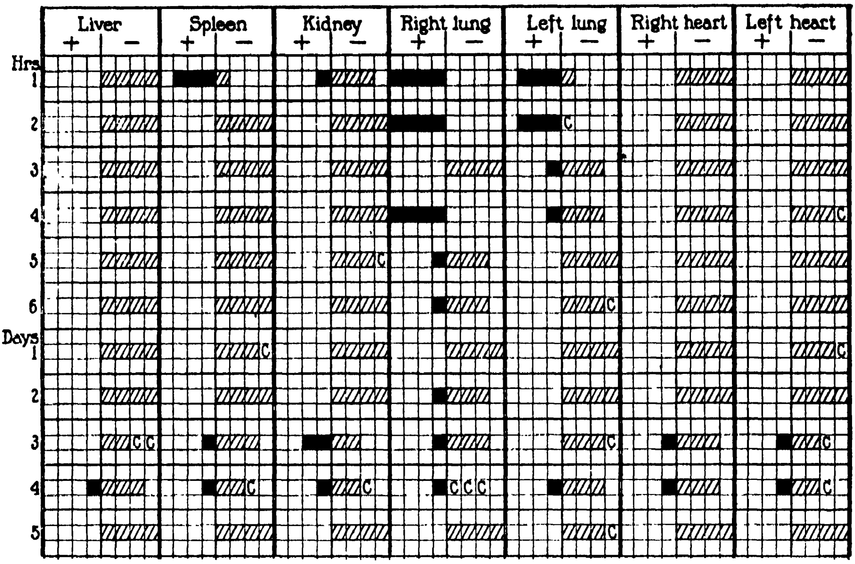
In all, 11 rabbits were exposed to from 1 to 10 inhalations of slightly virulent Type II pneumococci (SAv). Eight rabbits completed the course of 10 exposures. All these rabbits showed no evidence of infection.

Distribution of Rabbit Virulent Strain of Type II Pneumococcus (Sv)

In Text-fig. 3 is shown the distribution of rabbit virulent Type II (Sv) pneumococci in the organs of 44 sprayed rabbits which were killed at intervals after spraying. From the 24 rabbits killed in from 1 to 6 hours after their initial exposure, pneumococci were generally recovered from the periphery of the lungs for the first 2 hours but later they rapidly disappeared. During this same period pneumococci were only recovered in 3 rabbits from the spleen and in 1 rabbit from the kidney. Of the remaining 20 rabbits killed from 1 to 5 days following exposure, 2 or 10 per cent showed a general pneumococcus septicemia. In 2 other rabbits pneumococci were recovered from the lungs, and in 1 animal from the kidney. The results of this experiment do not markedly differ from those obtained in the case of the rabbits exposed to the slightly virulent strain of Pneumococcus Type II (SAv). A few rabbits may become infected following inhalations of rabbit virulent strain of Type II pneumococci (Sv). It further indicates that these virulent pneumococci do not disappear from the lung or organs as rapidly as do the avirulent organisms. If they persist, however, a generalised septicemia is more apt to be present.

Blood Cultures

Blood cultures were taken from 11 rabbits following their first exposure to a spray of rabbit virulent Pneumococcus Type II (Sv).



TEXT-FIG. 3. Distribution of Type II (Sv) pneumococci in organs following spraying. The black squares indicate organs in which cultures showed pneumococci to be present, the cross-hatched squares represent organs in which cultures remained sterile. "c" = contamination.

TABLE II

Days on Which Positive Blood Cultures Were Obtained Following a Single Exposure to a Pneumococcus II (Sv)

Rabbit No.	Days										Course
	1	2	3	4	5	6	7	8	9	10	
1	–	–	–	–	c	+	+				Died 8th day
2	–	c	–	–	–	–	–	–	–	–	Survived
3	c	–	–	–	–	–	–	–	–	–	"
4	–	–	–	–	–	–	–	–	+	–	"
5	–	–	–	–	–	–	–	c	–	–	"
6	+	+	+								Died 4th day
7	–	–	–	–	–	–	–	–	–	–	Survived
8	–	c	+	c	–	–	–	–	–	–	"
9	–	–	–	–	–	–	–	–	–	–	"
10	+	c	–	–	–	–	–	–	–	–	"
11	–	+	+	+	–	–	–	–	–	–	"

+ indicates a positive culture of pneumococcus; – = a sterile culture; and "c" = a contaminated culture.

As is seen from Table II, of the 6 rabbits which developed septicemia 2 or 18 per cent continued to have a positive blood culture until death. The other 4 rabbits had merely a transient septicemia and survived. In some instances, the septicemia could be detected during the first 24 hours, but in others the organisms were first recovered as late as the 6th or 9th day.

TABLE III

Fate of Rabbits after One to Ten Exposures to Inhalation of Rabbit Virulent Type II Pneumococcus*

No. of times exposed to virulent pneumococci	No. of rabbits exposed	No. dying	Pneumococcus recovered from heart's blood	Pneumococcus not recovered from heart's blood
1	50	14	12	2,
2	36	1	–	1
3	34	2	1	1
4	30	1	—	1
5	26	2	1	1
6	24	2	1	1
7	21	—	—	—
8	17	1	–	1
9	17	—	—	—
10	16	1	1	–

* The discrepancy between the total numbers and the sum of the numbers in the individual groups is due to the fact that certain of the animals that survived were used for intraperitoneal tests or for other experiments.

Mortality of Rabbits Following Exposure to Inhalation of Pneumococcus Type II (Sv)

Table III shows the number of normal rabbits which succumbed during the course of spraying with rabbit virulent Pneumococcus II (Sv). From this table it is seen that out of 50 rabbits 15 or 28 per cent died following the first exposure. Pneumococci were recovered from the heart's blood in 12 instances. Out of the total of 24 rabbits which died, in 16 instances the organisms were recovered from the heart's blood, and the cultures remained sterile in the other 8. Although care was exercised to open the spray boxes promptly 1 hour after spraying, these animals probably died of suffocation. This table further shows that the greatest number of deaths occur after the first exposure. It is difficult to explain, however, how some rabbits which have successfully survived 5, or even 9, exposures finally become infected.

The interval elapsing between the time of spraying and death from pneumococcus septicemia is shown in Table IV. From this it is seen that there is great irregularity in the time of death following the initial exposure. In fact 4 rabbits only succumbed on the 10th day. It is interesting to observe that in rabbits which had survived the first exposure, infection was delayed and death occurred from the 7th to 10th day after spraying.

In no instance was there any gross evidence of pneumonia, although serous and serofibrinous pleurisy and serofibrinous pericarditis were common.

TABLE IV

Number of Days Elapsing between Spraying and Death of Rabbits from Pneumococcus Type II (Sv) Septicemia

	Days									
	1	2	3	4	5	6	7	8	9	10
Spray I	–	1	2	2	–	1	1	1	–	4
“ III	–	–	–	–	–	–	–	–	1	–
“ V	–	–	–	–	–	–	1	–	–	–
“ VI	–	–	–	–	–	–	1	–	–	–
“ X	–	–	–	–	–	–	–	–	1	–

DISCUSSION

From the foregoing experiments it appears that the susceptibility of rabbits to infection following inhalation of the same type of pneumococcus is in direct proportion to the virulence of the strain used.

The non-virulent IIR pneumococci may be recovered from the periphery of the lungs for a period of 3 hours after spraying. After this time they are only occasionally recovered from any part of the body. The slightly virulent Type II (SAv) and virulent Type II (Sv) may likewise be recovered from the lungs for a few hours following exposure. But in the case of the more virulent strains, there appears to be an almost immediate general dissemination of the organisms throughout the body, as is evidenced by the recovery of pneumococci from the spleen. The organisms then disappear from the blood only to reappear after 48 hours. This reappearance is often in the form of a septicemia. The same phenomenon was noticed in the case of rabbits sprayed with virulent Type I pneumococci. It is unfortunate that the subsequent penetration of the inhaled pneumococci into the pulmonary tissues cannot be followed. But the great number of other organisms which normally inhabit the bronchi of rabbits, as Jones (3) has shown, renders cultural studies of the lungs difficult. Histological detection of pneumococci in the tissues is also unreliable when the organisms are present only in small numbers.

In all probability the majority of the organisms are destroyed either where they are first implanted or deeper in the lung tissue by the endothelial leucocytes or by poly-morphonuclear leucocytes. In any case, a few organisms occasionally filter through into the blood stream. Gaskell (4) believes that invasion of the blood stream by pneumococci probably always takes place in the early stages of an air-borne infection of the lung. The actual invasion of the blood stream must be much more common than is generally supposed. Not only are there probably few organisms free at any one time in the circulating blood but these few bacteria may even be within leucocytes and not in reality multiplying in the blood.

The occasional organisms which reach the blood are probably rapidly filtered out of the blood stream by the organs and locally destroyed. It is evident, however, that following the apparent disappearance of the pneumococci from the periphery of the lungs, they again appear, this time in the blood, and as a result may be recovered from all the organs. The point of this reinvasion is as yet uncertain.

The results of the blood cultures taken following the initial spraying parallel the distribution of pneumococci in the rabbits which were sacrificed. The cultures from the rabbits sprayed with avirulent degenerated non-virulent "R" forms pneumococci remained sterile; an occasional positive culture was obtained from the animals exposed to the slightly virulent Type II (SAv) organisms but no animals succumbed. Only in the case of the rabbits exposed to inhalations of rabbit virulent Type II (Sv) pneumococci did the more susceptible rabbits die of an overwhelming septicemia. But some rabbits were even able to overcome the septicemia following exposure to this rabbit virulent strain.

Following the initial spraying with a highly rabbit virulent Type II (Sv) pneumococcus the majority of susceptible rabbits died of an overwhelming septicemia without any attempt on the part of the body to localize the infection.

SUMMARY

1. The susceptibility of rabbits to inhalations of pneumococci varies in direct proportion to the virulence of the organism for rabbits.

2. When rabbits are exposed to a pneumococcus spray, irrespective of the virulence of the organism, the bacteria readily penetrate into the lower respiratory tract.

3. When rabbits are exposed to a spray of avirulent degenerated "R" form of pneumococcus, septicemia does not occur and pneumococci are seldom recovered from the liver, kidney or spleen.

4. When rabbits are exposed to a spray of slightly virulent Pneumococcus Type II (SAv) a non-fatal septicemia may occur and pneumococci may be recovered from the liver, kidney and spleen.

5. When rabbits are exposed to a spray of virulent Type II (Sv) pneumococci, septicemia may occur which in certain instances terminates fatally. Pneumococci may also be recovered from the liver, kidney and spleen.

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DEVELOPMENT OF AGGLUTININS AND PROTECTIVE ANTIBODIES IN RABBITS, AFTER INHALATION OF TYPE II PNEUMOCOCCI

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In preceding papers (1) it has been shown that mice and rabbits may acquire a high degree of immunity, following repeated inhalations of living pneumococci. In this work it was shown that the strain of Type I pneumococcus used produced a high mortality among the exposed rabbits and that many of the survivors have agglutinins and protective antibodies in their serum. In the present study it seemed interesting to determine the immunity response to less virulent strains. For this purpose a Type II pneumococcus was used. Pneumococcus Type II, however, is relatively avirulent for rabbits. One strain was transformed into the "R" form by repeated transfers in antipneumococcus Type II serum. The changes to the "R" form are accompanied by loss of capsule, type specificity, and virulence. This strain is referred to as Pneumococcus IIR. The stock culture of Pneumococcus Type II would cause a fatal septicemia in rabbits when injected intraperitoneally in doses of at least 0.1 cc. This strain is called Pneumococcus Type II (SAv). By repeated animal passages the virulence of this strain was raised so that an intraperitoneal injection of 0.000,01 cc. of broth culture would regularly kill rabbits. This virulent culture is designated as Pneumococcus Type II (Sv).

It has already been shown that rabbits are only susceptible to fatal infection by inhalations of virulent Type II pneumococci (Sv) (2). Although pneumococcus septicemia may be caused in an occasional rabbit following inhalation of the slightly virulent Pneumococcus Type II, death does not ensue. In the present paper is reported the development of (1) agglutinins and (2) protective antibodies in the blood serum of rabbits following repeated inhalations of avirulent IIR, slightly virulent Type II (SAv), and virulent Type II (Sv) pneumococci.

Method

Rabbits were placed in a large spray chamber similar to that already described (3) and exposed to a spray of pneumococci. 50 cc. of an 8 hour broth culture were used for each spraying. The animals were exposed at 10 day intervals. Before each spraying, a sample of blood was obtained from the ear vein of each animal. None of the normal rabbit sera contained agglutinins nor did the normal sera protect mice against intraperitoneal injections of 0.000,001 cc. of Type II pneumococcus.

10 days after each exposure the presence of agglutinins in the serum of exposed animals was determined by a modified thread reaction. To 1 cc. of rabbit serum diluted in normal salt solution was added 0.2 cc. of an actively growing broth culture of Pneumococcus Type II. The tubes were incubated for 2 hours in the water bath at 37°C., placed in the ice box overnight, and the reactions read the next morning. Agglutinins were recorded as present in the serum only when the reactions were positive in a dilution of at least 1:10.

The presence of protective antibodies in the blood of the sprayed rabbits was determined by the ability of 0.2 cc. of serum to protect white mice against intraperitoneal injection of 0.001 cc. of Pneumococcus Type II culture, of which 0.000,001 cc. killed a normal mouse within 48 hours. The rabbit serum and culture were administered simultaneously.

Pneumococcus IIR

Of the 17 rabbits which were sprayed from 1 to 10 times with "R" forms derived from Type II pneumococcus none died from pneumococcus septicemia. Not even the 8 rabbits which were exposed 10 successive times developed any demonstrable agglutinins for Type II pneumococcus.

Neither did the sera of any of these rabbits protect mice against intraperitoneal injections of 0.001 cc. of a virulent culture of Pneumococcus Type II. As is seen from Table I the sera of these 5 animals, however, showed evidence of slight protection as demonstrated by the presence of a low degree of protective antibodies. Even the slight amount of protection afforded by these sera is not constant.

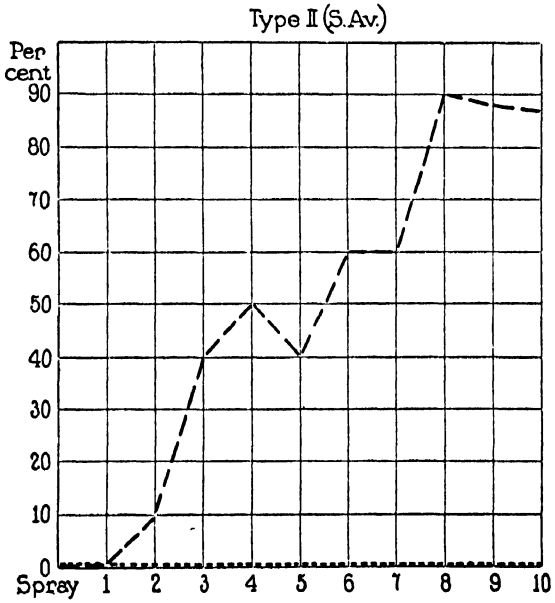
Pneumococcus Type II (SAv)

Of 11 rabbits sprayed from 1 to 10 times with the slightly virulent Pneumococcus Type II (SAv), none died from pneumococcus septicemia. Not even the 8 rabbits which had completed the whole course of 10 exposures developed any demonstrable agglutinins for Type II pneumococcus.

TABLE I

First Appearance and Degree of Protection Following Repeated Exposure to Pneumococcus IIR

Following exposure................	3	4	5	6	7	8	9	10
Rabbit 1..................	10^{-6}	—	10^{-6}	—	10^{-6}	10^{-6}	10^{-5}	
" 2..................	—	—	—	—	—	—	—	10^{-6}
" 3..................	—	—	—	10^{-5}	10^{-5}	10^{-6}	10^{-5}	10^{-4}
" 4..................	—	—	—	—	10^{-6}	10^{-6}	10^{-6}	—
" 5..................	—	—	—	—	—	—	10^{-6}	—



TEXT-FIG. 1. Comparison of presence of protective antibodies and agglutinins in rabbits following repeated inhalations of slightly virulent Type II (SAv) pneumococci.

- - - - - - - per cent of rabbits showing agglutinins in serum following spraying.

— — — — per cent of rabbits showing protective antibodies in their serum.

Protective Antibodies

In Table II is shown the number of rabbits which were repeatedly sprayed from 1 to 10 times with Pneumococcus Type II (SAv) and the number thus exposed of which the serum subsequently protected mice against intraperitoneal injection of 0.001 cc. of a virulent culture of Type II pneumococcus. From this table it is seen that protective antibodies were demonstrable in the sera of 1 of the rabbits after the 1st spray. Following each successive spraying, the percentage of rabbits showing protection steadily increased, until after the 8th spraying the serum of 90 per cent of the animals conferred passive protection on mice against at least 1000 lethal doses of virulent cultures. The development of protective antibodies is graphically shown in Text-fig. 1. The exposure following which the rabbit sera first protected mice is shown in Table III. From this it is seen that with each successive spray the number of rabbits in the sera of which protective antibodies were demonstrable progressively increased: Certain rabbits showed protective antibodies only after the 7th or 8th exposure.

TABLE II

Number of Rabbits Exposed to Pneumococcus II (SAv) and Number Yielding Serum Which Protected Mice against 0.001 Cc. of Pneumococcus Type II

No. of exposures	1	2	3	4	5	6	7	8	9	10
No. of rabbits	11	10	10	10	10	10	10	10	9	8
" " " whose serum protected	—	1	4	5	4	6	6	9	8	7
Per cent of rabbits whose serum protected	—	10	40	50	40	60	60	90	88	87

TABLE III

First Appearance of Protective Antibodies in the Serum of Rabbits Exposed to Inhalation of Pneumococci Type II (SAv)

No. of spray	1	2	3	4	5	6	7	8	9	10
No. of rabbits showing protective antibodies	—	1	3	1	1	1	1	1	—	—

Pneumococcus II (Sv)

Of 50 rabbits sprayed from 1 to 10 times with the virulent strain of Type II (Sv) pneumococcus, 12 or 24 per cent died of pneumococcus septicemia after the first exposure. As the more susceptible animals succumbed early, serum was obtained only from rabbits with the greatest natural resistance, individuals which had also acquired a certain degree of immunity, as the result of repeated exposures to live pneumococci.

TABLE IV

Relation of Development of Agglutinins to the Number of Exposures to Rabbit Virulent Pneumococcus II (Sv)

No. of exposures	1	2	3	4	5	6	7	8	9	10
No. of rabbits	38	34	32	29	23	22	21	17	16	15
" " " showing agglutinins	3	3	2	2	1	2	2	2	2	2
Per cent of rabbits showing agglutinins	7	8	6	6	4	9	9	11	12	13

TABLE V

First Appearance and Titre of Agglutinins Following Repeated Inhalations of Rabbit Virulent Pneumococcus Type II (Sv)

Following exposure	1	2	3	4	5	6	7	8	9	10
Rabbit 1	1–20	—	—	—	—	—	—	—	—	—
" 2	1–10	1–10	—	1–10	—	1–10	1–10	1–20	1–40	1–40
" 3	1–10	1–50	1–200*							
" 4	—	1–20	1–20	1–50	1–40	1–10	1–10	1–10	1–10	1–10

—, no agglutinins demonstrable in serum.

* Accidental death of rabbit following 3rd exposure.

In Table IV is shown the number of rabbits which were sprayed from 1 to 10 times whose serum showed agglutinins in dilutions of at least 1 to 10. From Table IV it is seen that 3 or 7 per cent of the 38 rabbits which survived the first exposure developed demonstrable agglutinins.

Only 4 rabbits developed agglutinins. In Table V are shown the exposures after which agglutinins first appeared and the titre of agglutinins following each subsequent spraying. Three rabbits showed agglutinins after the first exposure. Following the initial appearance of the agglutinins there is a tendency for the titre to rise after subsequent exposures. But in one instance the agglutinins disappeared, and in another became less marked.

Protective Antibodies

In Table VI are shown the number of rabbits that were sprayed from 1 to 10 times with rabbit virulent Pneumococcus II (Sv) and the number thus exposed the serum of which subsequently protected mice against intraperitoneal injections of 0.001 cc. of a virulent culture of Type II (Sv) pneumococcus. From this table it is seen that protective antibodies were demonstrable in the sera of 4 or 10 per cent of the rabbits after the 1st spray. Following each successive spraying the number of rabbits yielding serum which conferred passive protection increased, until after the 10th spraying the serum of 8 or 53 per cent of the animals conferred passive protection on mice against at least 1000 lethal doses of virulent culture. The spray following which the sera first protected mice is shown in Table VII.

TABLE VI

Number of Rabbits Exposed to Rabbit Virulent Pneumococcus Type II (Sv) and Number That Developed Protective Antibodies in Their Serum

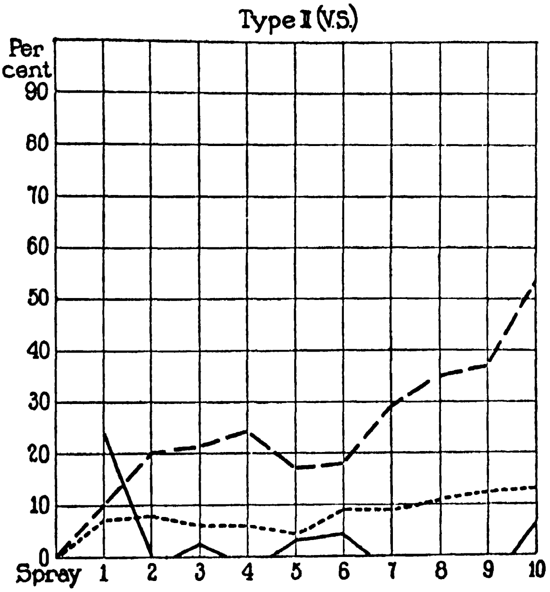
No. of exposures	1	2	3	4	5	6	7	8	9	10
No. of rabbits	38	34	32	29	23	22	21	17	16	15
" " " whose serum protected	6	7	7	7	4	4	4	6	6	8
Per cent of rabbits whose serum protected	10	20	21	24	17	18	29	35	37	53

TABLE VII

First Appearance of Protective Antibodies

No. of spray	1	2	3	4	5	6	7	8	9	10
No. of rabbits first showing protective antibodies	6	1	—	1	—	—	—	1	1	3

From Table VII it is seen that after the 1st exposure the sera of 6 rabbits showed protective antibodies. After successive sprays an occasional rabbit would first show protective antibodies. Protective substances were first demonstrated in the sera of 3 rabbits only after the 10th exposure.



TEXT-FIG. 2. Comparison of mortality and presence of protective antibodies and agglutinins in rabbits following repeated inhalations of virulent Type II (Sv) pneumococci.

———— per cent of rabbits dying with pneumococcus septicemia following successive sprayings.

- - - - - - - - per cent of rabbits showing agglutinins in serum following spraying.

— — — — per cent of rabbits showing protective antibodies in their serum.

Correlation of Agglutinins and Protective Antibodies

It is difficult to compare the relative titre of the sera at any one time because of the difference in the standards used. Whereas agglutinins were recorded as positive if present in serum concentration of 1:10, protective antibodies were noted only if they were present in concentrations sufficient to protect mice against a 0.001 cc. of virulent pneumococcus culture. If smaller doses of pneumococcus had been used, the presence of protective antibodies would undoubtedly have been detected earlier and the incidence of their occurrence would have been more frequent. However, when these antibodies were demonstrable under the conditions of this experiment, their presence was evidence of the capacity of the serum to confer a high degree of passive immunity on mice.

In 3 instances both agglutinins and protective antibodies occurred after the same spray; in another rabbit, yielding serum that contained agglutinins, protective antibodies appeared after the next exposure. In 9 instances protective antibodies occurred without demonstrable agglutinins in the serum and the sera of 7 rabbits, even after 10 exposures, contained no agglutinins or protective antibodies even sufficient to protect mice against 0.000,001 of a virulent Type II pneumococcus.

The relative occurrence of agglutinins and of protective antibodies in the serums of rabbits following inhalations of rabbit virulent strain of Type II (Sv) pneumococcus is graphically shown in Text-fig. 2. In this figure the mortality curve of rabbits dying from pneumococcus septicemia is also given. It is seen that after the 1st exposure 24 per cent of the rabbits died from pneumococcus septicemia. An occasional rabbit died following subsequent exposures. The sera of the rabbits dying after the 3rd, 5th and 6th exposures showed neither agglutinins nor protective antibodies. The serum of the rabbit dying after the 10th spray, however, protected mice against 0.01 cc. of pneumococcus culture. It is unfortunate that it is not known if the pneumococcus recovered from this rabbit was the original type specific organism used for spraying or a degenerated "R" form. The number of rabbits that developed agglutinins was never great, nor was the titre high. The proportion of rabbits showing agglutinins rose only slightly following successive exposures. On the other hand, there was a distinct tendency for the proportion of rabbits to develop protective antibodies with each successive spray. Following the 10th exposure 53 per cent of the total number of survivals showed the presence of these immune substances in their serum.

DISCUSSION

Although too few rabbits were used to give as definite results as after spraying with Type I pneumococcus, some definite results are indicated. It appears that the immunological reactions of rabbits following inhalations of the same type of pneumococci vary in direct proportion to the virulence of the organism. In the case of exposure to a non-virulent degenerated "R" strain derived from Type II pneumococcus no type specific antibody response was elicited. If rabbits are allowed to inhale slightly virulent Type II (SAv) pneumococcus, none succumb to infection, although a certain number develop a pneumococcus septicemia. As the result of successive spraying a large proportion of the animals developed protective antibodies in their serum but no agglutinins. Following the initial exposure to a rabbit-virulent Pneumococcus Type II (Sv) the greatest number of rabbits succumbed to a fatal septicemia. Certain rabbits developed a high degree of immunity as evidenced by the presence of agglutinin and protective antibodies in their serum. Why a larger proportion of rabbits exposed to the slightly virulent Pneumococcus Type II (SAv) developed protective antibodies than those sprayed with the virulent Pneumococcus Type II (Sv) is difficult to explain. The reaction of the rabbits to inhalations of the virulent Type II (Sv) culture parallels the results obtained when rabbits were sprayed with a rabbit-virulent Type I pneumococcus.

From the foregoing experiments it is certain that following inhalation of living virulent pneumococci of Type I or II rabbits develop a high degree of immunity. This immunity is probably induced by a few organisms penetrating the respiratory epithelium and entering into the body tissues. It has already been shown that rabbits may recover from a transient pneumococcus septicemia following inhalation of pneumococci. The great variations both in the time of first appearance and in the final titre of immune bodies are difficult to explain.

Among the factors which cannot be experimentally controlled are: first, the number of organisms which come to lodge within the respiratory tract following exposure to a bacterial spray; second, the number which are able to invade the tissues after implantation; and third, the final disposition of these bacteria in the animal body. In certain instances the multiplication of the invading organisms goes on unchecked until the death of the animal. In others a transient carrier state may occur with subsequent immunity responses. It is certainly significant that whereas the curve of incidence of protective antibodies steadily increases, the percentage of rabbits showing agglutinins does not materially change after the 5th spraying.

CONCLUSIONS

1. Following repeated inhalations of the degenerated non-virulent "R" forms of Type II pneumococcus, no type specific antibodies can be demonstrated in the serum of rabbits.
2. Following repeated inhalations of slightly virulent Type II (SAv) pneumococci, only protective antibodies can be demonstrated in the serum of rabbits.
3. Following repeated inhalations of virulent Type II (Sv) pneumococci, agglutinins and protective antibodies can be demonstrated in the serum of rabbits.
4. Following repeated exposures of rabbits to inhalation of pneumococci, the type specific response, evidenced by type specific protective antibodies and agglutinins, varies in direct proportion to the virulence of the culture used.

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REACTIONS OF RABBITS TO NON-HEMOLYTIC STREPTOCOCCI

III. A STUDY OF MODES OF SENSITIZATION

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In previous papers (1, 2) we have described the various types of tissue response of rabbits to inoculation with non-hemolytic streptococci. It was shown that reactions following reinfection were conditioned by the route of the original inoculation: if it had been into the tissues—especially into the skin—the subsequent reaction was that of hypersensitiveness, or hyperergy; if into the blood stream, it was that of immunity. For the sake of completeness two other types of reaction were recorded: that of normal animals, which in the majority of instances was followed by a secondary reaction, and that of cachectic rabbits, in which inoculation resulted in a soft, pale lesion smaller than that of normal animals, and not followed by a secondary reaction.

Variability in the response of apparently normal rabbits to inoculation with streptococci is well recognized; and because of this some investigators feel that this species is unsuitable for the study of experimental streptococcus infections. Under ordinary experimental conditions this factor necessitates the introduction of a sufficient number of animals into each experiment to permit of conclusions on a statistical basis. While such a basis is always requisite in biological research the advantages of having to depend upon it as little as possible are obvious.

During our investigations the desirability became more and more evident of ascertaining the best method of rendering a large majority of rabbits satisfactorily hypersensitive to non-hemolytic streptococci in the shortest possible time. This communication gives the details of some of the experiments planned with such an end in view.

EXPERIMENTAL

Both green-producing and indifferent strains of non-hemolytic streptococci of previously demonstrated sensitizing capacity have been used. From an original stock culture, preserved in the frozen and dried state, sub-stocks in rabbits' blood broth were prepared every 6 to 10 weeks. As the work progressed it seemed that old stock cultures in blood broth gradually lost their sensitizing capacity; more recently, therefore, the blood broth stocks have been renewed from the dried culture every 4 to 6 weeks. The inoculum for the animals was a culture grown for from 18 to 24 hours in 2 per cent rabbits' blood broth to which 0.05 per cent dextrose was added. Both Douglas tryptic broth and beef infusion broth have been used. If the sediment of more than 0.1 cc. of whole culture was to be injected in one place the culture was centrifuged and sufficient of the supernatant fluid was discarded so that 0.1 cc. of the residue represented the amount of growth selected for injection; 0.1 cc. of whole culture was injected if that was the quantity desired: and in the case of the smaller inocula the culture was diluted with broth or Ringer's solution so that 0.05 cc. represented the amount injected. It was found that the intracutaneous injection of 0.05 cc. of some samples of broth gave early evanescent, edematous lesions in some highly sensitized rabbits and occasionally in normal controls; but these lesions never became indurated as did those following injection of culture. Dilution of the culture with Ringer's solution did not give rise to these false reactions; hence only this diluent has been employed recently. All skin lesions were measured daily until marked diminution in size was evident, and the volumes were estimated as previously described (1). The ophthalmic and lethal tests were carried out as previously noted (1); the dose of culture employed for the lethal test was the centrifugate of 4 cc. of blood broth culture per kilogram of rabbit.

To obtain as nearly uniform conditions as possible animals of corresponding weight and color were distributed among the different groups. During the past year we have found that Chinchillas and New Zealand Reds have more consistently become hypersensitive after intradermal inoculation and, on the whole, have had more suitable skins; hence these varieties have been used when obtainable.

Attempts were first made to determine which was the most efficacious in sensitizing animals: one, two or four intracutaneous injections, each with the sediment of 5 cc. of culture. Lesions produced with such large doses frequently broke down and discharged their contents within a few days. Another disadvantage in injecting such large doses was the failure, at times, of the resulting lesions to show clear-cut secondary reactions, whereas those produced by 10^{-1} cc. or 10^{-2} cc. in the same animals gave distinct evidence of such reactions.

With equivocal secondary reactions in animals treated with large inocula alone it was necessary to resort to ophthalmic and lethal tests to ascertain the state of reactivity. In two or three different experiments the results were so variable that no satisfactory conclusions could be drawn from the exclusive use of these large inocula. It was then observed that rabbits became increasingly sensitive following initial intracutaneous inoculation with the sediment of 5 cc., 1 cc., 10^{-1} cc., and 10^{-2} cc. of blood broth culture if they subsequently received repeated injections of 10^{-1} cc. and 10^{-2} cc. at 10-day intervals. In carrying out this procedure so many areas of skin were inoculated that little remained available for subsequent observations; hence it became desirable to devise other modes of sensitization in which less cutaneous damage was inflicted.

Experiment 1.—Eighteen rabbits were each inoculated intracutaneously in five areas with the sediment of Culture V110A/0/13, as follows: 5 cc., 5 cc., 10^{-1} cc., 10^{-2} cc., 10^{-4} cc. They were then divided into three equal groups, A, B and C. On the 8th day each animal of Group A received a subcutaneous focus of 5 cc. of agar infected with the sediment of 5 cc. of culture. The animals of Group B were inoculated intramuscularly with 10^{-1} cc. and 10^{-2} cc. from a similar culture every 5 days from the 5th to the 40th day, and those of Group C with the same doses intracutaneously. The degree of skin reactivity of Group A animals was tested with 10^{-4} cc. of culture on the 1st, 15th, 25th, 35th and 40th days, and that of the animals in the other two groups on the 1st, 10th, 20th, 30th and 40th days. The ophthalmic test was applied to the right eye of all animals on the 13th day and to the left on the 43rd day. During this interval one rabbit from each Group B and C had died. On the 48th day all of the animals except two of Group A received a subcutaneous focus of 5 cc. of agar infected with sediment from 5 cc. of culture of Streptococcus V110A. Six fresh rabbits comprising Group D received similarly infected agar foci. All animals were then allowed to go untreated until the 101st day, an interval of 53 days, when their right eyes were retested in the usual manner and their skin reactivity to inoculation with 10^{-4} cc. of homologous culture was measured. The comparative intensity of the cutaneous reactions to 10^{-4} cc. of culture is shown in Chart 1, and that of the ophthalmic reactions is shown in Table I.

The results clearly indicate that after the animals had been rendered hypersensitive by means of the initial skin inoculation the condition was satisfactorily maintained when an infected focus was present. At that time, however, we did not appreciate the influence of repeated intracutaneous test inoculations with 10^{-4} cc. of culture in maintaining

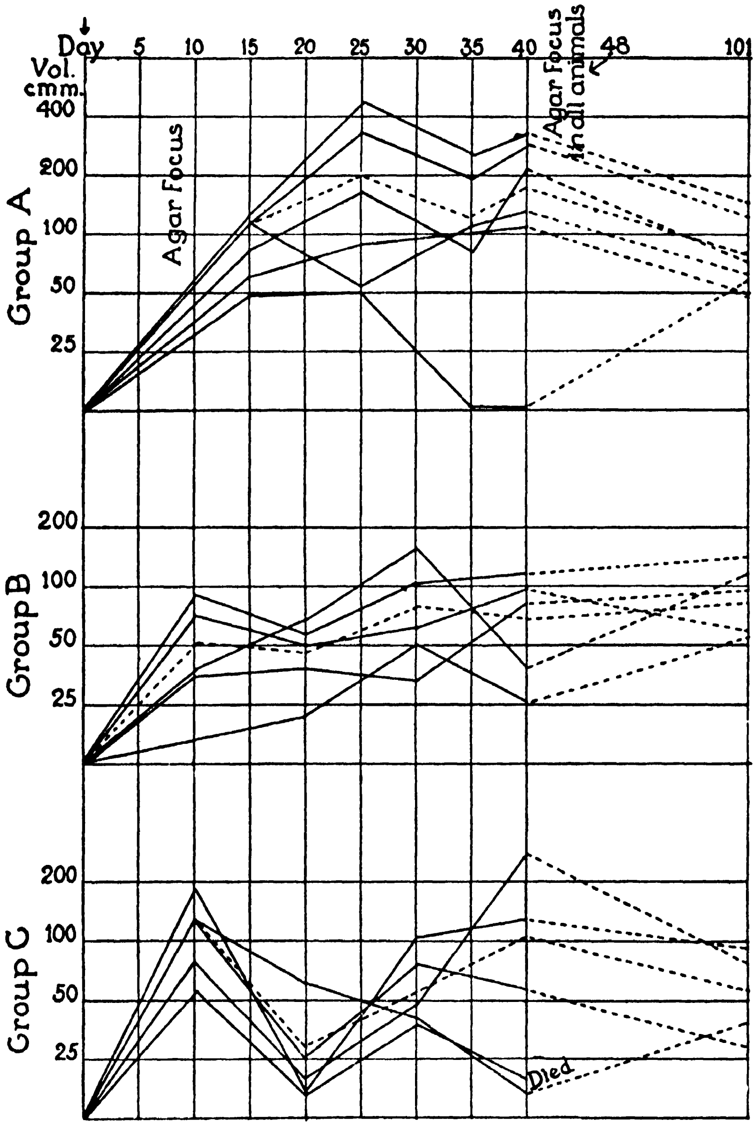

Chart 1. Comparative intensity of cutaneous reaction in rabbits sensitized in different manners.

the hypersensitive state (see Experiment 2). Yet the animals of Groups B and C also received similar small intracutaneous inoculations repeatedly at 10-day intervals concomitantly with the course of

10^{-1} cc. and 10^{-2} cc. doses, without developing a comparable degree of hypersensitiveness. Indeed, it seems probable from the appearance of the curves of Group C that the repeated intracutaneous inoculation with these larger doses may have resulted in a temporary diminution of sensitiveness at about the 20th day. This opinion was confirmed by a parallel diminution in the size of the cutaneous lesions resulting from inoculations with 10^{-1} cc. and 10^{-2} cc. of culture. Other experiments have shown a similar wave-like variation in intensity of reactions in animals receiving repeated intradermal inoculations of large doses of *Streptococcus viridans*.

In this experiment the skin lesions resulting from 10^{-1} cc. inocula were usually as large after the third and subsequent inoculations as were those produced by the sediment from 5 cc. at the time of the original inoculation. It is possible that these large cutaneous foci containing much necrotic tissue and many bacteria may have had less sensitizing effect than the smaller areas produced by 10^{-4} cc. of culture. This possibility is rendered more probable from comparative consideration of Group B. These animals, although receiving the major portion of the inocula into their lumbar muscles, always showed cutaneous lesions at the site of the needle punctures; lesions that were often of the same size as followed intracutaneous inoculation with 10^{-4} cc. of culture. They, therefore, were under the sensitizing influence of two small intracutaneous inocula every 5 days and of three similar additional ones every 10 days. Under these combined muscular and cutaneous lesions, however, the animals seemed to remain at a constantly lower level of sensitivity than existed in Group A, where the repeated sensitizing cutaneous stimuli were very small.

At the end of 6 weeks the average cutaneous and corneal sensitiveness of Group A was distinctly greater than at the end of the 2nd week, and also higher than that of Groups B and C, in which it was relatively the same as in the 2nd week. While on the 13th day none of the Group C animals gave a positive ophthalmic reaction, one did so by the 7th week (see Table I).

The effect of an infected agar focus implanted on the 48th day was to maintain or increase the corneal sensitiveness of nearly all the animals by the 101st day, even though the cutaneous reactivity of Groups A and C had fallen off to a certain degree. It is true that the

TABLE I

Comparative Intensity of Ophthalmic Reactions in Rabbits Sensitized in Different Manners

Group	Rabbit No.	13th day Rt. eye				43rd day Left eye				101st day Rt. eye			
		Degree of reaction	Days between inoculation and appearance of:			Degree of reaction	Days between inoculation and appearance of:			Degree of reaction	Days between inoculation and appearance of:		
			Congestion	Veil	Pannus		Congestion	Veil	Pannus not observed		Congestion	Veil	Pannus
A	R832	+	3	3	5	+++	1	1		+++	1	1	2
	R833	+	2	3	7	+++	1	1		++++	1	1	2
	R834	–	–	–	–	–	–	–		±	1	1	0
	R835	++	1	2	5	+++	1	1		+++	1	1	3
	R836	±	3	4	–	+	2	3		+++	1	2	5
	R837	?	2	–	–	+++	1	1		+++	1	1	2
B	R838	+	+	1	–	+	2	2		–	–	–	–
	R839	–	–	–	–	–	–	–		+++	1	1	3
	R840	–	–	–	–	–	–	–		+++	1	2	5
	R841	+	1	1	5	++	1	2		+++	1	1	6
	R843	++	1	1	5	±	1	2		+++	1	1	3
C	R844	–	–	–	–	++	2	2		++	1	1	6
	R845	–	–	–	–	–	–	–		+++	1	1	5
	R847	–	–	–	–	–	–	–		±	1	1	0
	R848	–	–	–	–	–	–	–		†			
	R849	–	–	–	–	–	–	–		++	1	1	5
D	R878									–	–	–	–
	R879									–	–	–	–
	R880									++	7	7	9
	R881									±	8	8	0
	R882									–	–	–	–
	R883									++	4	4	6
E	Controls												
	R897									–	–	–	–
	R898									+	10	10	11
	R899									++	10	10	12
	R900									–	–	–	–

† Animal died.

– = negative reaction.

± to ++++ = varying intensity of reaction.

right eye, which had been inoculated on the 13th day, was the one tested on the 101st day and the effect of this first inoculation must be considered. But among eight of the surviving animals in which the first inoculation of the right eye did not result in keratitis, the second—on the 101st day—was followed by distinct and intense reactions (see Table I).

These reactions, moreover, appeared promptly as compared with those of Groups D and E. Group D animals had been sensitized 53 days previously by an infected subcutaneous agar focus, without any preliminary cutaneous inoculations. Only one-half of this group developed positive ophthalmic reactions at all, and these were all weaker than in the first three groups, and were also slower in appearing. The members of Group E were introduced for the first time at the end of the experiment, to serve as controls of the activity of the culture at that time; like the animals of the other groups they received intracutaneously 10^{-1} cc., 10^{-2} cc., and 10^{-4} cc. of culture on the day their corneae were inoculated. Two developed secondary cutaneous reactions and shortly afterwards showed weak but distinct ophthalmic reactions. These two animals illustrate well that for the development of keratitis a state of hypersensitiveness is necessary; for even though their eyes were inoculated early, they showed corneal lesions only after the development of this state as indicated by the secondary reactions. The five groups of animals show clearly, as judged by their ophthalmic reactions, how variations in the degree of hypersensitiveness may be conditioned by differences in the method of previous inoculation.

Experiment 1 indicated that in most rabbits a high degree of hypersensitiveness could be obtained in 35 to 40 days by the following method: initial inoculation of several large doses of green streptococci, followed by the introduction of a single infected agar focus, and thereafter by repeated intracutaneous inoculations with 10^{-4} cc. of culture at 10-day intervals. In two subsequent experiments carried out in almost the same manner, but without skin testing every 10 days, such a satisfactory degree of hypersensitiveness was not obtained. In fact the proportion of highly sensitive animals was very disappointingly small. The only differences between the two series of experiments consisted in the absence of small intracutaneous inoculations every 10 days in the second, and in the seasons of the year in which the two were carried out. Variations in sensitizing capacity of culture must also be considered.

Experiment 2 was therefore planned to determine whether one infected subcutaneous agar focus, or multiple intracutaneous foci from repeated single inoculations with 10^{-4} cc. of culture every 10 days, or a combination of the two, was most effective in maintaining the hypersensitive state.

Experiment 2.—Each of 64 rabbits was inoculated intracutaneously with the sediment of 5 cc., 5 cc., 10^{-1} cc., and 10^{-4} cc. of blood broth Culture V110A/0/6. On the 8th day they were divided into four groups. Into each animal of Group A (27 rabbits) and Group B (25 rabbits) was placed a subcutaneous focus of 5 cc. of melted agar inoculated with the sediment of 5 cc. of 24-hour blood broth culture. Groups C and D, containing 6 rabbits each, received no agar foci. Groups A and B were larger because it was hoped by production of agar foci to obtain many highly sensitive animals for a subsequent experiment. On the 11th day the right cornea of each rabbit was inoculated in the usual manner. The number and percentage of positive ophthalmic reactions is indicated in Table II. The proportion of animals in Groups A, B, and D, which developed secondary reactions was approximately the same; but Groups A and B had a much higher proportion of positive ophththalmic reactions than did Groups C and D. It seemed, therefore, that the implanting of agar foci at the time when the animals were becoming hypersensitive increased the sensitiveness of the eyes; this is a confirmation of similar observations in Experiment 1. The animals of Groups A and C were inoculated intracutaneously with 10^{-4} cc. of homologous culture on the 11th, 21st and 31st days. On the 42nd day the left eye of all the rabbits was tested, and all animals received 10^{-4} cc. of culture intracutaneously. The volumes of the resulting skin lesions were computed from daily measurements; the average for each group is given in Table II. Five days later the cutaneous reactivity of all the rabbits was again tested with 10^{-4} cc. of each of two cultures, one of which had been grown from an old blood broth stock preserved in the ice-box, while the other had been inoculated from a stock recently derived from the original frozen and dried material. The lesions induced by these two cultures were quite comparable in size; the average volume is recorded in Table II.

At the end of 6 weeks each of the four groups had been subjected to different sensitizing conditions. In so far as the secondary reaction is an indicator of sensitivity all groups on the 11th day seemed to be about equally hypersensitive; but the eye test showed that Group D was less so than the others. While at this early period only one animal of Group C had developed a marked, and one a slight, keratitis, three subsequent intracutaneous injections of 10^{-4} cc. of culture at 10-day intervals apparently so increased the corneal hypersensitiveness of animals of this group that when tested on the 42nd day, three developed strongly positive ophthalmic reactions. In Group A, on the other hand, the degree of corneal sensitiveness was found to have decreased, even though the animals had received the same intracutaneous treatment as those of Group C. But by contrast the continual sensitizing effect of these small repeated inocula is illustrated in Groups B and D, in which they had been omitted: Group B on the 42nd day gave approximately one-third the number of positive eye reactions that it did on the 11th day, and Group D failed entirely to develop sufficient hypersensitiveness for demonstration by the ophthalmic test. The comparative intensities of the skin reactions in the various groups on the 42nd and 47th days also showed that the repeated production of cutaneous foci with only 10^{-4} cc. of culture

TABLE II

Summary of Effect of Agar Foci and Repeated Intracutaneous Inoculation with 10^{-4} cc. of Culture on Maintaining the Hypersensitive State in Sensitized Rabbits

Group	No. of animals	1st day	8th day	11th day				11th day 21st " 31st "	42nd day			47th day
		Sediment of 5 cc., 5 cc., 10^{-1} cc. and 10^{-4} cc. intracutaneously	Agar focus inoculated with sediment of 5 cc.	Secondary reaction		Ophthalmic reaction		Intracutaneous reinoculation 10^{-4} cc.	Ophthalmic reaction		Intracutaneous inoculation 10^{-4} cc. Average vol. in cmm.	Intracutaneous inoculation 10^{-4} cc. Average vol. in cmm.
				+	−	+	−		+	−		
A	27	+	+	21 78%	6	19 76%	8	Yes	12 44%	15	75	102
B	25	+	+	17 68%	8	18 72%	7	No	6 24%	19	52	64
C	6	+	0	3 50%	3	2 33%	4	Yes	3 50%	3	77	133
D	6	+	0	4 67%	2	0 0%	6	No	0 0%	6	34	62

was more favorable for the continuation of the hypersensitive state than was the production of a single subcutaneous agar focus. The stronger reactions of all of the animals on the 47th day as compared with the 42nd indicate either that the culture used for testing was more potent or that the animals were more reactive. As the two different cultures used for testing on the 47th day gave reactions of approximately the same intensity, it is probable that the cutaneous sensitivity of all of the rabbits had beenelevated by the small cutaneous inoculation 5 days previously. The experiment in all of its phases indicates clearly that after animals are made hypersensitive, this state can be continued or increased by cutaneous foci induced by doses of streptococci which are incapable of stimulating macroscopically visible lesions in normal animals.

In practically all previous experiments it had been assumed that a first inoculation of the sediment of 5 cc. of culture was necessary for the development of the hypersensitive state; in other words, that a large amount of bacterial substance was the important factor in giving the initial impulse. But control groups in a number of experiments, such as Group E in Experiment 1, had shown that distinct hypersensitiveness might follow the production of three lesions with 10^{-1} cc., 10^{-2} cc. and 10^{-4} cc. of culture respectively. Moreover, in a comparison of the sensitizing capacities of a large number of strains of indifferent streptococci (3) it was found that nine small lesions often led to a marked degree of hypersensitiveness. It seemed advisable, therefore, to determine whether the total dosage of streptococci injected intracutaneously or the number of lesions produced was the more important in inducing a condition of hypersensitiveness, provided the lesions were all made at one time.

Experiment 3.—Five groups of 4 rabbits each were inoculated intracutaneously in a varying number of places with different doses of indifferent Streptococcus Q186A/0/6, as shown in Table III. The number of lesions varied between 1 and 11 and the total dosage was the centrifugate of either 0.222 cc., 5.0 cc., or 5.222 cc. of a 20-hour blood broth culture. All of these lesions were measured daily to detect secondary reactions; and on the 14th day the comparative hypersensitiveness of the animals was tested with intracutaneous inocula of 10^{-2} cc., 10^{-4} cc., and 10^{-6} cc. of a homologous culture, and the ophthalmic test was applied to each. A new group (F) of normal controls was introduced at this time to test the activity of the culture.

Two rabbits of Group A sickened of *B. lepisepticum* infection; hence comparison with the others is somewhat vitiated. But the two survivors developed secondary reactions, and showed lesions on reinfection comparable with those of the other groups. Moreover, the results of the rest of the experiment do not indicate that these Group A animals would necessarily have been less sensitive than any of the others. Group E, the members of which received only a single injection of 5 cc. of centrifugate, was the most poorly sensitized. Group C, on the other hand, which received approximately only 1/20th of the dose used in the other animals but divided amongst six areas, developed hypersensitiveness in as many members as either Group B or D. The latter group, however, showed the largest lesions following reinoculation.

TABLE III

Comparative Sensitizing Effect of Different Doses of Streptococci Distributed in a Varying Number of Lesions

Group		Amount of inoculum					Each animal		Total with secondary reaction	Test 14th day			
										Positive ophthalmic reaction	Average maximum volume retest on 14th day		No. of animals showing reaction with
		5 cc.	1 cc.	10^{-1} cc.	10^{-2} cc.	10^{-3} cc.	Total lesions	Total dose			10^{-3} cc.	10^{-4} cc.	10^{-3} cc.
A	No. of lesions	1	...	2	2	2	7	5.222	2*	1	367**	96**	2
B	induced by	...	5	2	2	2	11	5.222	3	1	375	79	2
C	inocula of	...	...	2	2	2	6	0.222	3	2	325	55	3
D	respective	...	5	...	...	...	5	5.0	3	2	532	101	4
E	size	1	...	...	...	...	1	5.0	1	1	387	36	2
F													
Controls		...	...	...	...	...	...	...	...	0	85	0	0

* Only 2 rabbits of Group A survived until the 14th day.

** Figure indicates average for the group.

The culture used in this experiment was capable of producing in normal animals lesions about 1 cm. in diameter with doses of 10^{-3} cc., but no macroscopic lesions with 10^{-4} cc. In other experiments it has been observed that unless a particular strain could evoke fair sized lesions with doses of at least 10^{-1} cc. or 10^{-2} cc. it was practically useless in eliciting secondary reactions or in inducing a state of hypersensitiveness of sufficient intensity to be detected by the ophthalmic test. One may conclude, therefore, that if with any suitable strain lesions of about 10 mm. diameter are produced by relatively small doses, a number of these small lesions will more readily induce a hypersensitive state in rabbits than will a single lesion made by injecting a much larger quantity.

The demonstration that intracutaneous injection of non-hemolytic streptococci at 10-day intervals, in doses 1/10th the size necessary to produce minimal macroscopic lesions in normal animals, either maintained or enhanced the hyperergic state in rabbits already somewhat hypersensitive, made it appear that possibly it was the amount of tissue injured in a peculiar manner rather than the quantity of bacterial substance which furnished the direct stimulus for hypersensitization.

The time (30 to 60 days) required by this method to render the animals sufficiently hypersensitive for further experimental procedures made it desirable to determine whether the same principle might not be applied to secure a more rapid sensitization of the animals.

Experiment 4.—The microorganism, P64, used in this experiment, was a green-forming streptococcus, which in doses of 10^{-2} cc. of blood broth culture induced in normal rabbits lesions of 10 to 15 mm. in diameter and about 1 mm. in height; these usually persisted for 1 to 3 days. Macroscopic lesions were usually not visible at the site of 10^{-3} cc. inoculation; but at the sites of injection of 1 cc. and 10^{-1} cc. there occurred long tracking zones of redness and edema which gradually disappeared, and left areas in which secondary reactions were most likely to occur. Each rabbit of two groups was inoculated intracutaneously with 1 cc., 10^{-1} cc., 10^{-2} cc., and 10^{-3} cc. of a 24-hour blood broth culture. Group A animals were simply observed in the usual manner; but those of Group B received during the next 2 weeks 11 injections each of 10^{-3} cc. of similar cultures; these inoculations were made daily with the exception of the 6th and 13th days; and each resulting lesion was measured daily until it diminished in size. On the 15th day the ophthalmic test was applied to the left eyes, and on the 22nd day all of the rabbits were inoculated intravenously with the sediment of equivalent amounts per kilo body weight of 24-hour blood broth culture. The results are summarized in Table IV.

It is obvious that daily inoculations for 2 weeks induced distinct hypersensitiveness in all of the Group B animals, while those of Group A receiving merely the original inocula failed to become sufficiently sensitive to respond to any of the three tests used to detect this state. It is not to be expected that such a clear cut difference between the two groups would always appear, for with other strains a considerable degree of hypersensitiveness has often been induced in some animals by a single series of inoculations similar to or smaller than that used in Group A. With this strain, however, we were fortunate in obtaining conditions favorable for the illustration of the advantage of the added daily inoculation of small doses.

TABLE IV

Comparative Sensitizing Effect of Initial Inoculum and a Similar Inoculum Followed by Daily Inoculation

Group	Rabbit No.	Secondary reaction		Reaction from 10^{-3} cc. inoculation		15th day ophthalmic reaction	22nd day lethal reaction
		10^{-0} cc.	10^{-1} cc.	Begun	Maximum		
A	R2126	0	0	...	...	0	0
	R2127	0	0	...	...	0	0
	R2129	0	0	...	...	0	0
B	R2130	± 10th day	++++ 12th day	4th day	10th day	++	+
	R2131	+ 12th day	+++ 7th day	3rd day	9th day	++	+
	R2132	++++ 12th day	++++ 15th day	4th day	10th day	?	S*

* Sick, but recovered.

Another point brought out in this experiment was the early onset of hypersensitiveness, detected by measuring the 10^{-3} cc. lesions in Group B (see Chart 2). Whereas the first two or three areas inoculated with this dose showed little or no gross evidence of infection, those following the third or fourth inoculation developed distinct maculopapules, and each subsequent injection induced a lesion of greater volume than the previous one. The maximum reaction occurred in areas inoculated on the 9th or 10th days, or about the time of appearance of the secondary reactions at the sites of the original larger inoculations. Then the intensity of the reactions following reinoculation seemed to diminish somewhat, a suggestion that an "immune state" was being superimposed on the pre-existing hyperergic condition. Similar diminution in reactivity has been observed by Julianelle (4) in sensitizing rabbits with pneumococcus vaccines.

Still another advantage is suggested for this mode of sensitization. The recording of the amount of reaction occurring at the site of each reinoculation discloses, by the end of the first week, the probable capacity of the animal to develop a satisfactory state of hypersensitiveness. This point is brought out by comparing Rabbit R2132 with the other two of the group (see Chart 2). The degree of reaction was slow in increasing, and was always small as compared with that of the other two animals; the secondary reaction was correspondingly slow in developing; the ophthalmic reaction was equivocal; and intravenous inoculation merely caused the animal to sicken without leading to death. In subsequently applying this mode of sensitization we have been able to determine within 7 to 10 days that certain animals were showing the cachectic type of response or failing to become satis-

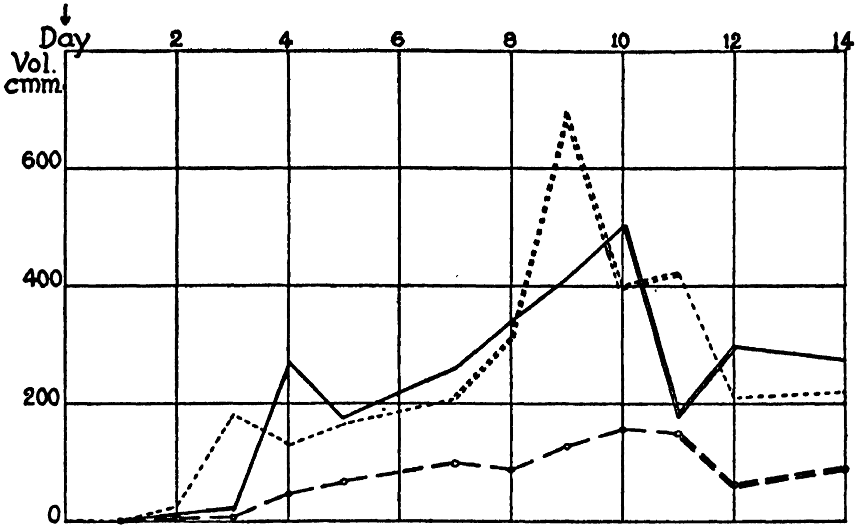

CHART 2. Volume of lesions resulting from daily inoculation with 10^{-3} cc. of Streptococcus P64.

Double lines indicate time of appearance of secondary reaction at site of original inoculations.

factorily hypersensitive, and thus to eliminate them from any series to be used for comparative studies.

From Experiment 4 it was not clear whether daily inoculations with small doses of streptococci for 2 weeks without the initial larger inocula might not have been as efficacious as the two combined; hence additional experiments were performed to answer this question.

Experiment 5.—Streptococcus V110A was used as the sensitizing strain. The animals of Groups A and B were inoculated in a manner similar to those in Experiment 4; but those of Group C received only daily inoculations of 10^{-3} cc. amounts over a period of 2 weeks. Ophthalmic tests did not reveal sharply defined differences amongst the various groups; but in this experiment, as well as in Experiment 4, the findings at autopsy after comparable intervals following lethal tests showed the most severe and widespread lesions in animals of Group B, which had received daily small inoculations in addition to an initial series of larger doses; the members of Group C, which had been sensitized with daily inoculations alone, showed somewhat more extensive lesions than those of Group A which had received simply the initial inoculations.

Comparison of the average volumes of the reactions from repeated 10^{-3} cc. inocula showed that in the 2-week period the animals of Group B had somewhat larger lesions; hence one may conclude that in this period the strong initial stimulus combined with daily inoculation was more efficacious than the daily small inoculations alone.

Experiment 6.—In Experiment 6 the reactions to daily inoculation of 10^{-3} cc. of blood broth culture of *Streptococcus viridans* V110A were followed for 4 weeks. In Group B initial inocula of 1 cc., 10^{-1} cc., and 10^{-2} cc. were given; in Group C these were withheld, while in both groups single daily injections of 10^{-3} cc. were made. The average of the two groups is shown in Chart 3.

In all animals a wave-like rise and fall of cutaneous reactivity was observed. The curve rose most rapidly in the first 10 or 12 days; then there was a fall followed by a second peak about the 20th day, following which a second fall occurred.[1] All animals of Group B then showed a stationary or falling trend; in one this was very marked as though the animal were becoming "immune." It was impractical to carry the experiment further because most of the good available skin had been utilized. This experiment demonstrates, however, that a high degree of sensitivity can be attained by repeated daily inocula of relatively small doses of non-hemolytic streptococci, when carried out over a prolonged period.

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[1] A somewhat similar wave-like variation in reactivity to repeated intracutaneous inoculation is recorded by Krause and Willis in tuberculous guineapigs (*Am. J. Tuberculosis*, 1926, **14**, 316).

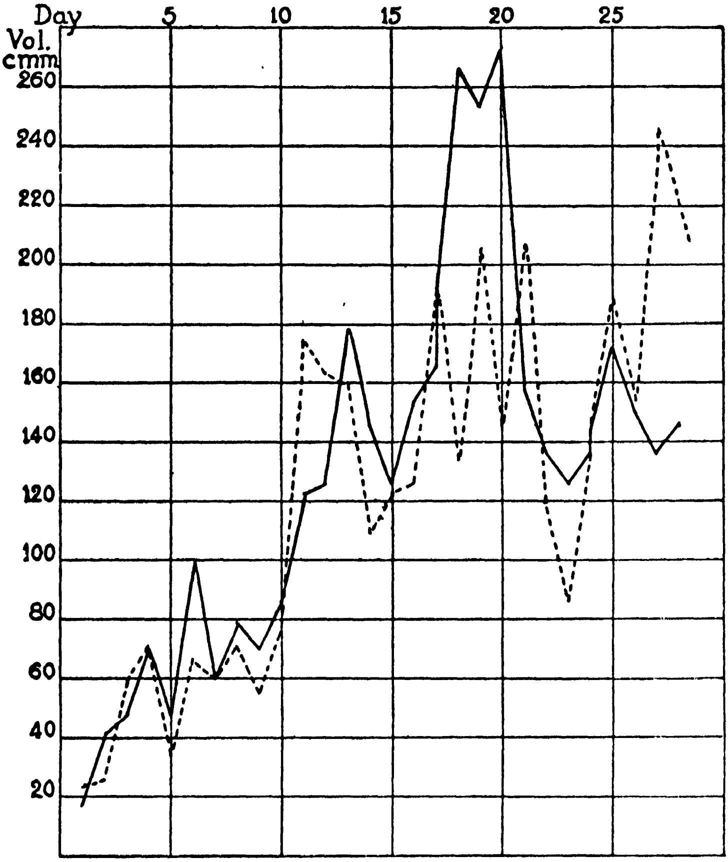

Chart 3. Comparative intensity of reactions at site of daily inoculations of 10^{-3} cc. of Streptococcus V110A.

Solid line, Group B received original inoculation of 1, 10^{-1} and 10^{-2} cc. followed by daily inoculation of 10^{-3} cc. Broken line, Group C received daily inoculation of 10^{-3} cc. only.

The tendency for the lesions to be less marked during certain periods in animals receiving daily inocula suggested that possibly the hypersensitive state might be temporarily depressed as a result of too frequent inoculation; and in addition Experiment 2 suggested that after a certain degree of hypersensitivity had been attained, less frequent inoculations would serve to maintain or heighten this condition. Experiment 7, therefore, combined certain features of the two methods.

TABLE V

Comparison of Degree of Hypersensitiveness of Corneae after 2 and 9 Weeks

No. animals	Strain	After 2 weeks' sensitization. Ophthalmic reaction			After 7 weeks' additional sensitization 10^{-3} cc. and 10^{-4} cc. each week			
					Ophthalmic reaction			Lethal test. No. died
		−	±	+ to ++++	−	±	+ to ++++	
3	Q155	...	1	2	...	1	2	2*
3	Q167B	3	...	...	1	...	2	3
2	R1	2	...	...	1	...	1	2
4	R9	...	2	2	...	...	4	4
2	RF156	...	...	2	...	...	2	2
3	RF162B	3	...	...	2	...	1	0
3	A49	1	2	...	...	...	3	3
Total, 20		9	5	6	4	1	15	16
Per cent........		45	25	30	20	5	75	80

* The other animal of the group rendered very sick, but recovered. Autopsy findings showed typical changes.

Experiment 7.—Seven different strains of streptococci, six indifferent and one green-forming, were employed; each rabbit received but a single strain, with a distribution of strains among groups as shown in Table V. The inoculation on the first day consisted of 1 cc., 10^{-1} cc., 10^{-2} cc., and 10^{-3} cc. of blood broth culture; thereafter daily inocula of 10^{-3} cc. were given for 2 weeks, at the end of which time the right corneae were inoculated with homologous streptococci. For the next 7 weeks each animal received 10^{-3} cc., and 10^{-4} cc. of homologous culture every 7 days. The left eyes were then tested; and 1 week later all animals were inoculated intravenously with the usual lethal test dose of the homologous strain. The results are summarized in Table V.

It is evident that the 7 weeks' additional treatment greatly increased the hypersensitiveness of the entire group. While somewhat more than one-half of the animals showed some degree of corneal sensitivity at the end of 2 weeks, in one-fourth it was only slight. Seven weeks later, however, the number of strong ophthalmic reactions had more than doubled. The lethal test, moreover, indicated that all of the animals except those inoculated with Strain RF162B had been rendered very hypersensitive by the prolonged treatment; this was a much larger number of positive lethal reactions than would have been expected at the end of 2 weeks in a group of animals showing so few strongly positive ophthalmic reactions. In fact, if the animals inoculated with Strain RF162B be deleted from the experiment, and the one severely but not fatally shocked animal of the Q155 group be included, it can be said that all of the rabbits inoculated with six of the strains showed at the end of 9 weeks marked general hypersensitivity. The RF162B group illustrates the necessity of employing suitable strains in order to obtain satisfactory sensitivity, for although these animals were treated in identically the same manner as the others, their skin reactions were always poor and none of them was rendered sufficiently sensitive to sicken after intravenous inoculation.

DISCUSSION

Several interesting points have emerged during the progress of this work. At the beginning it was thought that the degree of hypersensitiveness would be more or less proportional to the size of the initial dose. This was to be expected from the work of Roemer (5) Krause (6) and others on hypersensitiveness in experimental tuberculosis, and of Bloch (7) and his co-workers in trichophyton infections. But it soon became evident that, within certain limits, the initial production of multiple lesions with smaller doses of non-hemolytic streptococci was more likely to lead to marked hypersensitivity than that which followed a single lesion induced by larger amounts; furthermore, the most satisfactory hypersensitiveness followed repeated inoculations with small doses of the microorganisms. In a number of instances these doses were too small to induce macroscopic lesions in normal animals.

The results of infection of animals with single inocula of non-hemolytic streptococci and with tubercle bacilli are not exactly comparable. In the first instance, as the microorganisms are so quickly killed they do not multiply long at the site of inoculation and are disseminated to other areas for only a short time. In the second case they grow and are carried to distant organs, where they again increase and produce lesions; hence in the first instance relatively few local lesions are produced, while in the second case multiple and disseminated injuries occur. Repeated small inoculations with streptococci, therefore, probably reproduce more nearly the conditions following a single inoculation with tubercle bacilli. This view point is substantiated by the results of implanting agar foci infected with streptococci. Such foci harbor living streptococci for a period of from 3 to 4 weeks, but even after becoming sterile they presumably contain disintegrated bacterial sensitizing products for long periods. Nevertheless these foci have not proven as efficacious in increasing the sensitive state as have repeated inocula of small size. This suggests that the kind of lesion rather than the amount of bacterial substance is the important factor. Indeed, lesion—tubercle production—is the *sine qua non* for the development of tuberculin allergy; and it is highly suggestive that Zinsser and Mueller (8) found that the production of multiple skin tuberculous lesions in rabbits yielded the best conditions for passive transfer of tuberculin allergy. They stress the rôle of the focus in the induction of "allergy;" but later Zinsser (9) thought that the peculiar manner in which the bacteria are disintegrated was the important factor. Whatever may be formed from the bacteria in a focus, our experiments indicate that only exceedingly small amounts of the substance are necessary to increase the hypersensitive condition, once the initial stimulus has been provided for the development of that state.

Experience with various infections indicates that there are two types of reaction towards reinfection with microorganisms—that of hypersensitivity, and that of immunity. That the two are often intimately linked is indicated by the opinions of most workers in this field. But there is a growing tendency to regard the two processes as not necessarily interdependent (Boquet and Nègre (10), Swift and Derick (1, 2), Rich (11)). One problem of experimental investigation is to determine the best manner of imposing these two types of reaction, each independently of the other, on different animals, in order to study their respective rôles in the general subject of resistance. Julianelle (4) noted that rabbits sensitized by intracutaneous injection of vaccines of pneumococci showed increasing lesions up to a certain time;

there was then a decrease which was associated with the appearance of immune bodies in the serum. Panton and Valentine (12) observed that rabbits receiving repeated inoculations with living staphylococci eventually showed decreasing reactions to large inocula and increasing reactions to small ones, another example of concomitant hypersensitiveness and immunity. Both groups of observers employed relatively large inocula; although the latter stated that hypersensitiveness could be induced by small doses.

From a theoretical standpoint it might be expected that when relatively large amounts of bacterial substances are acting within an animal's body there is more possibility of wide distribution and accompanying immunity. With small amounts, there is simple focal lesion-production, and attending hypersensitivity. Our experiments indicate that repeated small focal lesions usually lead to increasing general tissue hypersensitivity without local evidence of immune tissue response. Unfortunately the relative avirulence of the non-hemolytic streptococci makes it impossible to test the general immunity of rabbits so sensitized; but we have observed that rabbits may be highly sensitized and show little or no serum agglutinin against the sensitizing microorganism, and little precipitin against its nucleoprotein. Contrasted with this may be cited the observations of Fleischer and Meyer (13) that following intraperitoneal injections of vaccines prepared from *B. abortus* or *B. typhosus*, guinea pigs would yield high agglutinins against the homologous bacterium without giving abortin or typhoidin reactions.

The influence of chronicity of infection as a sensitizing factor is indicated by a number of our experiments; and again let it be emphasized that the dose of bacteria sufficient to increase the hypersensitive state was too small to induce macroscopic lesions in normal animals. Thus in the hypersensitive animal there are set up conditions favorable for the augmentation of this state in the presence of a minimum dose of antigen. Klinge (14) has recently emphasized the importance of chronicity in the production of certain peculiar reactions to coagulable protein; while the observations of Dienes (15) suggest that the local condition of the tissue into which a coagulable protein is introduced may also determine the subsequent type of reaction to that protein. Both factors doubtless have important influences, but in order to evaluate them the importance of dosage of antigen must be considered more carefully than in the past. In experimentally inciting the hypersensitive state (tuberculin-like allergy or hypersensitiveness of infection) large doses of bacteria have usually been employed; but in natural infections characterized by so-called "allergy"—better "hyperergy"—the infecting dose of microorganisms is usually small, and presumably the inciting of new lesions at a distance from the initial lesions is usually effected by relatively few germs which doubtless multiply to a certain extent at the site of metastatic implantation. But in syphilis, which is a classical example of changing "allergic" reactions, it is noteworthy that the most destructive lesion, the gumma, usually arises only after the spirochetes have been acting in the body for a number of years; and at times antecedent evidences of infection—in other words, lesions—may have been so small as to escape detection. Other clinical examples of "allergic alteration" of tissues might be cited to show that in nature the hypersensitive state is induced or maintained by small lesions existing or reproduced over considerable periods of time.

SUMMARY

The most satisfactory method thus far found for the induction and maintenance of a high degree of hypersensitiveness—"allergy," "hyperergy"—against non-hemolytic streptococci consists in the repeated production of small focal lesions with minimal doses of bacteria.

After a preliminary sensitizing period of about 2 weeks' duration with either large initial, or small multiple daily inoculations, the later foci need be produced only at 7 to 10 day intervals.

Chronicity of low-grade infection appears to be an important factor in the attainment of a high degree of hypersensitiveness.

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INFECTIOUS MYXOMATOSIS OF RABBITS

Observations on the Pathological Changes Induced by Virus myxomatosum (Sanarelli)

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Plates 34 to 37

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In 1927, a preliminary report (1) was made concerning certain features of a disease designated by Sanarelli as infectious myxomatosis of rabbits. The purpose of the present communication is to amplify the previous report and to record some results of subsequent work.

Infectious myxomatosis is a malady indigenous to rabbits of South America and was first described by Sanarelli (2) in 1898. The disease, as he saw it, was characterized by the presence of nodules in the skin in the neighborhood of the eyes, nose, mouth, ears, and genitalia. A conjunctivitis, accompanied by a profuse purulent discharge, resulted from the involvement of the skin around the eyes. The disease ran a rapid course, death of the animals usually occurring within 1 or 2 weeks after infection. Upon palpation the tumors were firm and on section revealed a gelatinous consistency. Histologically they were found to be comprised of gelatinous material, large stellate cells, and blood vessels. The lymph nodes and spleen were enlarged, and, in histological preparations of the latter, nests of the large stellate cells were observed. The presence of the virus was demonstrated in the secretions from the eyes, in the tumors, in the various organs, and in the blood. Besides spreading spontaneously, the malady proved to be transmissible by experimental inoculation. Many kinds of animals, including man, were inoculated. The rabbit, however, was the only susceptible host found. Sanarelli was unable to cultivate the etiological agent on artificial media, nor was he able to see it by means of the microscope. In view of these facts, he concluded that *Virus myxomatosum* is similar in nature to rabic virus.

Since Sanarelli's original communication only 10 papers (see bibliography) dealing with the infectious myxoma of rabbits have appeared. A detailed review of these reports, with the exception of Findlay's (11), was made by Hobbs (10) in 1928. Therefore, only the results of previous work that are particularly interesting or that have a bearing on the experiments to be presented at this time will be described.

Moses, Hobbs, and Findlay have shown that the myxoma virus is capable of passing through filters impervious to ordinary bacteria. No worker has been able to cultivate the etiological agent on artificial media in the absence of living host cells. The rabbit is the only known susceptible host. Even the wild hares (*Lepus braziliensis*) of Brazil are resistant to the virus (Moses, 1911).

Splendore, 1909, reported the presence of trachoma-like bodies in the tumor cells. In 1911, Moses stated that he was unable to confirm Splendore's observations concerning these bodies. Aragão, 1911, recorded his observations of certain bodies, *Chlamydozoa myxomae*, within the hypertrophic nuclei of the tumor cells. In 1927, this investigator repudiated his work concerning the nuclear inclusions and stated that the etiological agent is represented by small round granules, *Strongyloplasma myxomae*, situated within the cytoplasm of infected cells. Rivers, 1927, reported that he was able to find large granular acidophilic inclusion bodies in the cytoplasm of epithelial cells covering the myxomatous masses. Hobbs, 1928, confirmed Rivers' observations concerning the presence of these inclusions in the epithelial cells. Lipschütz, 1927, described within the cytoplasm of the swollen tumor cells, the presence of many small bodies which, inasmuch as they seemed to differ from chlamydozoa and strongyloplasms, he designated *Sanarellia cuniculi*. Findlay, 1929, failed to confirm Rivers' observations regarding the presence of cytoplasmic inclusions in epithelial cells. He was able, however, to find Lipschütz's *Sanarellia cuniculi* in the tumor cells.

Although Sanarelli in 1898 described the myxomatous masses in the skin as tumors and found that in infected animals the spleen and lymph nodes were enlarged and that at times an orchitis occurred, certain workers have been unable to confirm his observations. For instance, Aragão, 1927, stated that the myxomatous swellings are not true tumors but remarkable collections of oedema due to an infectious agent. Furthermore, he observed no involvement of the lymph nodes and spleen. Findlay, 1929, reported that "the nodular lesions are thus due, not to an active proliferation of the tissue elements, but simply to the myxomatous changes in the tissues." Moreover, he found "no enlargement of the lymph-glands or spleen." Also, according to him, "the testicles, apart from congestion, were normal even when the scrotum was affected."

From the above brief review of the reports dealing with infectious myxomatosis of rabbits, it is obvious that many conflicting statements and opinions exist. Nevertheless, the disease is so characteristic and so fatal that there is no doubt but that all of the investigators studied the same malady. In fact, many of them were dealing with the same strain of virus, which originally came from the Oswaldo Cruz Institute in Brazil.

In a preliminary report, 1927, I described for the first time certain changes observed in epidermal cells covering myxomatous masses induced by *Virus myxomatosum*:

> Upon microscopic examination the first change noted in the epidermal cells is an increase in their size. Then, small pink, granular areas appear in the cytoplasm. These areas rapidly increase in size and frequently involve most of the cytoplasm. In the center of the acidophilic masses, blue, round or rod-shaped bodies are often seen. . . . The disease process in the epidermal cells progresses until there is complete dissolution of the cells. At this time distinct vesicles appear in the epidermis. . . .

Because of the involvement of the epidermis, a fact that had not been observed previously, I raised the question in my preliminary note as to whether I was dealing with more than one virus. Since that time work on the myxoma has been continued and now it seems advisable to record certain observations that may be of interest.

Methods and Materials

Virus.—The myxoma virus was obtained by Dr. C. E. Simon from Dr. A Moses of the Oswaldo Cruz Institute, Brazil. In May, 1926, Dr. Simon sent the virus to Dr. A. Carrel who gave it to me for study.

Methods of Inoculation.—Animals were inoculated epidermally, intradermally, subcutaneously, intravenously, and intranasally. A few animals were allowed to contract the disease through contact with infected rabbits. Except for the tumors that arose at the sites of inoculation, the disease picture was the same following all types of inoculation.

Cultures.—Infectious blood and bits of the tumors and different organs were tested for sterility by means of the usual aerobic and anaerobic cultures. No bacterium of etiological significance was encountered. In approximately 50 per cent of the rabbits that died of the myxoma, *P. lepiseptica* was obtained from some organ or tissue. Sufficient animals, however, with sterile tumor masses, blood, and organs were studied to convince one that the changes to be described in this paper were not induced by *P. lepiseptica*. In working with the myxoma, one should endeavor to use rabbits that are not carriers of *P. lepiseptica*.

Fixation and Staining.—Tissues used for histological studies were obtained from rabbits sacrificed by the intravenous injection of air. The tissues were fixed in Zenker's (5 per cent acetic acid) fluid and stained with eosin and methylene blue and according to Giemsa's method.

EXPERIMENTAL

The course of infectious myxomatosis and the histological findings are not materially influenced by the manner in which the disease is contracted. When the virus is rapidly passed in series from rabbit to rabbit, however, it kills the animals so promptly that sufficient time does not elapse for the formation of large and characteristic lesions. Therefore, if large tumors and metastases are desired for study, a virus that has been stored for a long time should be employed.

During a period of 4 years, approximately 100 rabbits have been used for various studies of the myxoma virus. Of these animals, 25 have been carefully autopsied and the tissues have been examined histologically. The results of each autopsy will not be given in detail. The findings in different tissues and organs will be described and the relative frequency of the involvement of these organs and tissues will be noted.

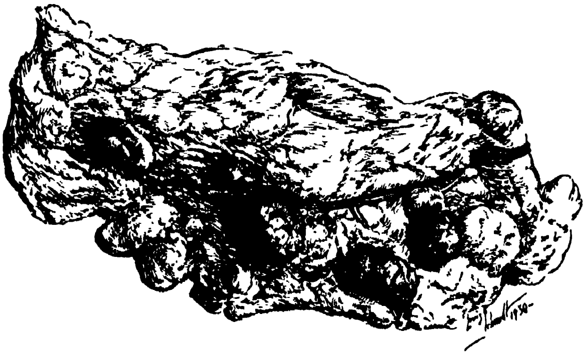
Skin

In every susceptible animal the epidermis, corium, and subcutaneous tissues were involved. The appearance of primary and secondary lesions in the skin is illustrated in Figs. 1–7. Such lesions are elevated, round or oval masses, the centers of which may take on a purplish color. Upon palpation the masses are firm and at times have the consistency of cartilage. The affected skin around the genitalia, however, frequently presents the appearance of oedematous tissue (Figs. 6, 7). If the lesions have progressed sufficiently, evidences of vesiculation can be found (Figs. 1, 5). In fact, fluid can be obtained from the vesicles by means of capillary pipettes. If the animals survive long enough, the vesicles are replaced by crusts which cap the tumor nodules (Fig. 5).

On sectioning the tumors one finds them firm, and hard to cut. The epidermis is thickened or shows evidences of vesiculation. The corium and subcutaneous tissues consist of a tough, pinkish, gelatinous material freely supplied with blood vessels. The tumors may be attached to the underlying muscles.

Histological examination reveals the following course of events in the epidermis. The cells increase in number and size (Fig. 9). Then small acidophilic granules appear in the cytoplasm. These rapidly increase in number and eventually replace the major portion of the normal cytoplasm. Among the acidophilic granules round or rod-shaped blue bodies may be seen (Figs. 21, 23). The nuclei become swollen or vacuolated (Fig. 23) and the chromatin is fragmented. Finally the cells undergo dissolution and vesicles appear in the epidermis (Figs. 10, 11).

In the involved corium and subcutaneous tissues an amorphous-looking material is seen in which large stellate or polygonal cells (Figs. 12, 24, 25), many polymorphonuclear leucocytes, and multinucleated cells (Figs. 12, 14) are found. The nuclei of the polygonal cells are swollen, and the chromatin is fragmented (Fig. 25). Dividing cells and mitotic figures are not numerous, but they do occur. Many of the so-called tumor cells are strikingly phagocytic. The cytoplasm of these cells contains a large number of granules. Whether some of the granules are similar to those described by Lipschütz and Findlay is not known. It seems, however, that the majority of the bodies represents not virus particles, but ingested material. The myxomatous masses are abundantly supplied with blood vessels, many of which are surrounded by the large polygonal cells (Fig. 12). At times the endothelial cells of the capillaries also seem to be involved (Figs. 12, 13) and are not unlike the large cells seen in other parts of the tumor.



TEXT-FIG. 1. Chain of axillary lymph nodes draining site of primary inoculation of the skin with myxoma virus. The glands were hypertrophic, hemorrhagic and firm. Histological examination of the 4 larger glands revealed the fact that the lymphocytes had been completely replaced by "myxoma" cells. Natural size

In the epidermis and in the corium affected by the virus it appears that more than a myxomatous metaplasia of the cells already present occurs. Evidences of growth and destruction of cells are found.

Lymph Glands

Examination of the axillary and popliteal lymph glands was a routine procedure of each autopsy. If the animal is inoculated in the skin over the lateral surface of the thorax and upper part of the abdomen, it is usual to find the axillary glands on the same side involved. Glands in the other axilla and in the popliteal spaces, in a great many instances, also show evidences of involvement. A marked degree of hypertrophy is the first change noted; at times the increase in size is tenfold. Then discrete red areas appear on the surface of the glands. Finally the nodes become very firm and hemorrhagic throughout (Text-fig. 1).

Histological examination reveals that the early changes in the lymph glands are characterized by an increase in the lymphatic tissue, *i.e.*, lymphocytes. Then in the lymph sinuses around the periphery of the glands phagocytic cells make their appearance (Fig. 18), many of which are multinucleated. Soon the lymphocytes begin to disappear, and, if sufficient time elapses, the glands become devoid of such cells. At this stage of the disease, the large lymph nodes consist of cells resembling those found in the subcutaneous myxomatous nodules (Fig. 19). These cells are not normal; they have hypertrophic nuclei with fragmented chromatin, and in their cytoplasm different kinds of granules are present, many of which seem to have been ingested. On studying the sections one obtains the impression that these cells arise from the reticulum of the glands. Mitotic figures are not numerous. Nevertheless, they do occur. If the cells that comprise these "metastases" or altered lymph glands arise from the reticulum, their number can be accounted for only upon the assumption that a multiplication of the cells of the reticulum takes place under the stimulus of the virus. In addition to the type of cells just described, many polymorphonuclear leucocytes are seen.

Spleen

The spleen may be enlarged or it may be of normal size. On histological examination nests of large stellate cells are not infrequently observed. In two rabbits, many of the blood vessels in the spleen were surrounded by myxomatous tissue (Figs. 17, 20) composed of cells similar to those described in the "metastases" in the lymph glands. Around each myxomatous nodule was a compact ring of cells, many of which seemed to be spleen cells crowded together by the growth of the myxoma. Other cells in this dense mass doubtless represented the results of host reaction to the infection.

Lungs

As a rule, lesions characteristic of the myxoma were not observed in the lungs. Occasionally a pneumonic process caused by *P. lepiseptica* was encountered. In sections of the lungs from one rabbit, however, numerous myxomatous nodules (Fig. 15) were seen in the vicinity of blood vessels and bronchi. The myxomatous tissue was similar in nature to that observed elsewere in the body. Moreover, the epithelial cells of the bronchi in close proximity to the myxomas showed evidences of hyperplasia, and, in the cytoplasma of these abnormal cells, changes similar to those seen in epidermal cells were noted.

Inasmuch as the myxoma virus is regularly found in the secretions from the eyes and nose and in the blood, it is difficult to state in what manner the virus that caused the lesions in the lungs obtained entrance.

Testicles

In the majority of the rabbits the scrotal skin is affected, but it is unusual for the testicle to be attacked by the virus. In 4 rabbits, however, myxomatous changes in the interstitial tissue of the testicle accompanied by a necrosis of the tubules was encountered.

Epididymis

The epididymis (Fig. 8) is involved more frequently than is the testicle. Within the connective tissue, myxomatous masses similar to those in the subcutaneous tissue are found. In one rabbit the epithelial cells lining different parts of the epididymis showed intracellular changes similar to those described in the epidermal cells, *i.e.*, an increase in number and size, and the presence of acidophilic cytoplasmic inclusions (Figs. 16, 22).

Tunica Vaginalis

It is not unusual to find hemorrhagic myxomatous nodules in the tunica vaginalis (Fig. 8).

Ovary and Uterus

Autopsies were performed on 3 female rabbits inoculated with myxoma virus. No lesions were found in the ovaries and uterus. If a large number of infected female rabbits were examined, it is possible that lesions might be encountered in these organs.

Pancreas, Liver, Kidney, Adrenals

No myxomatous nodules were observed in the pancreas, kidney, liver and adrenals.

Concerning the Presence of More than One Virus

The peculiar changes in the epidermis that accompany the myxomatous masses in the underlying tissue induced me to raise the question in my preliminary note (1) whether the observed pathological picture resulted from the combined activity of two viruses. Findlay (11), failing to find the changes in the epidermis described by me, again raised the question as to whether I was dealing with two viruses, one of which is indigenous to American rabbits and is not found in British rabbits. Furthermore, he suggested that one of the agents may be so labile that it is unable to withstand shipment from America to England.

During the last 4 years, I have tried in many ways to demonstrate the presence of more than one virus in the myxoma material and have been unable to do so. Vaccine virus and Virus III have been eliminated as possible contaminants by passage of the myxoma through rabbits immune to these agents. Fowl-pox also is not a contaminant, inasmuch as an emulsion of the myxomatous tissue will produce no disease in fowls. The myxomatous material is innocuous for mice, rats, guinea pigs, and monkeys. Therefore, if two viruses are present in the myxomas, both are specific for the rabbit.

In view of the fact that different viruses are not equally stable, an emulsion of myxomatous tissue in 50 per cent glycerol, and citrated infectious blood were stored on ice for 1 and 2 years respectively in the hope of eliminating in this manner one of the viruses in case two were present. Lesions resulting from inoculations with these materials showed the phenomena in the epidermis that gave rise to the question regarding the presence of two viruses. Therefore, if there are two viruses in the material with which I have been working, then both are extremely resistant to aging and storage or a large number of the rabbits that I have inoculated during the past 4 years have been carriers of the second or contaminating virus. The facts that I have adduced do not conclusively exclude the possibility of the presence of two viruses. Nevertheless, in view of them, such a possibility seems unlikely.

DISCUSSION

The fact that maladies induced by viruses are characterized by hyperplasia and necrosis was discussed in a previous paper (12). In the majority of the virus diseases both of these phenomena are observed. In some, however, hyperplasia may play the important rôle, while in others necrosis dominates the picture. Such a lack of balance between the growth and destruction of tissue accounts for the fact that the activity of some viruses gives rise to certain kinds of tumors, *e.g.*, Rous' sarcoma, while the operation of others lead to vesicular lesions, *e.g.*, variola and foot-and-mouth disease.

Many of the viruses are highly species specific. Virus III attacks only rabbits; the salivary-gland virus is active only in the guinea pig; hog cholera is infectious only for swine; each polyhedral disease has its specific caterpillar host; the tumor-forming activity of the Rous sarcoma seems to be limited to chickens; certain mosaic viruses produce pathological changes in one kind of plant. This marked degree of specificity, however, is not exhibited by all viruses. For instance, vaccinia and rabies may be induced in many kinds of animals.

A close relation exists between the viruses and host cells. This fact is emphasized by the lack of evidence to demonstrate that these active agents are capable of multiplication in the absence of living susceptible host cells, and by the presence in many instances of specific inclusion bodies in the affected cells. The relation between the viruses and cells may be not only close but specific in that certain types of cells alone are directly injured by the disease-inciting agent. For example, in rabies and poliomyelitis, it appears that nerve cells are the susceptible elements. Such cell specificity however, is not observed in vaccinia and herpes febrilis, diseases in which cells of ectodermal and mesodermal origin are involved.

The ease with which virus diseases spread from one host to another varies. Some are contagious, *e.g.*, variola and foot-and-mouth disease; others are only inoculable, *e.g.*, rabies and Rous' sarcoma; while yet others are capable of being transferred only by means of grafts, as is the case with certain infectious chloroses of plants.

The infectious myxoma of rabbits described in the present paper is one of the first maladies placed in the virus group. Moreover, when the available facts are closely examined, it is found to be one of the most interesting and characteristic of the lot. It is highly species specific, contagious, attacks cells of ectodermal and mesodermal origin, and causes hyperplasia and necrosis—the predominance of necrosis in the epidermis leads to vesicles, while the preponderance of hyperplasia in the subcutaneous and other tissues results in tumor-like masses. Moreover, inclusion bodies are found in epithelial cells involved. Finally, the causal agent is filterable, has not been cultivated in the absence of living host cells, and is very resistant to the action of certain chemicals and to long periods of storage.

Infectious myxomatosis, acquired spontaneously or induced experimentally, regularly exhibits foci of metastatic activity of the causal agent. These are always seen in the skin, particularly around the eyes, mouth, nose, and genitalia. The lymph glands draining the site of inoculation are usually involved. Other lymph nodes also may be affected. The epididymis and testicle with its tunic are not infrequently attacked. In an occasional animal, many metastases occur around the blood vessels of the spleen and rarely multiple metastases are found in the lungs. Inasmuch as the virus is always found in the blood, the word metastases, when used in connection with this disease, does not necessarily mean that multiple lesions arise through the transportation of affected cells from one part of the body to another. The metastases described above probably represent the results of the activity of the virus operating in different parts of the animal.

There is no adequate explanation of the fact that Hobbs and I found changes in the epidermal cells associated with cytoplasmic inclusions, while Findlay in England observed none. Nor is it easy to understand why only a few workers have noted involvement of the lymph glands. In any event, the results of the work reported at the present time indicate that the rabbits in New York over a period of 4 years when infected with *Virus myxomatosum* as a rule exhibited involvement of the lymph glands.

The exact nature of the stellate, polygonal, or myxomatous cells is not known. Lipschütz, (9) speaks of them as histiocytes, while Findlay (11) describes them as "hypertrophied connective-tissue cells." Furthermore, the nature of the inclusions in the epithelial (1) and connective tissue cells (9, 11) is still an open question, as is the case with many of the inclusion bodies in the virus diseases.

I can not agree with the statement of Findlay (11) and Aragão (8) that no active proliferation of tissue elements results from infection with the myxoma virus. The evidence obtained from my work is convincing that both growth and destruction of tissues occur in this disease. Of course the cells evidencing multiplication may be those already present in the tissues. Moreover, a myxomatous change may take place in these cells as a result of the virus activity, but such an occurrence does not alter the fact that an increase in the number of cells also results from the operation of the virus and that this increase plays a part in the formation of the nodules or tumor-like masses. These facts, however, are not proof that the myxomatous masses are true neoplasms similar to cancer in man. In fact, some investigators are not convinced that the exact relation of Rous' sarcoma to true neoplasms has been determined.

In certain respects the myxoma of rabbits resembles the Rous sarcoma of chickens. In others, however, it is quite different. If, as some believe, the Rous sarcoma appears to be more closely related to true neoplasms than to diseases induced by highly contagious agents, then the myxoma, upon further study, may serve to bridge the gap between the Rous tumor and other virus maladies, and to indicate that, after all, no great difference exists between tumor-forming viruses and those causing vesicular or destructive lesions.

SUMMARY

The virus of infectious myxomatosis of rabbits (Sanarelli) induces multiple lesions in the skin, lymph glands, tunica vaginalis, epididymis, testicle, spleen, and lungs.

Growth and destruction of cells in the epidermis overlying the myxomatous masses leads to the formation of vesicles. Cytoplasmic inclusions are found in affected epidermal cells. Occasionally, similar inclusions are seen in other involved epithelial cells. The nature of the inclusions is an open question.

In the myxomatous masses situated in the subcutaneous and other tissues, evidences of alteration and growth of certain cells are observed.

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EXPLANATION OF PLATES

PLATE 34

FIG. 1. Primary "tumor" surrounded by secondary nodules. Several vesicles cap the primary growth.

FIG. 2. Multiple primary tumors induced by rubbing the virus on the freshly shaved skin. The central portions of some of the nodules reveal a purplish tint.

FIG. 3. Involvement of the eyelids accompanied by a white, purulent discharge.

FIG. 4. Metastatic nodule in lip.

FIG. 5. Metastatic nodule in skin over shoulder. The growth is capped by a vesicle undergoing desiccation.

FIG. 6. Oedema around the vaginal orifice.

FIG. 7. Oedema of prepuce and scrotum.

FIG. 8. Four metastatic nodules, three in the tunic, one in the epididymis.

PLATE 35

FIGS. 9, 10, 11. Involvement of epidermis characterized by hyperplasia of the epithelial cells followed by necrosis resulting in vesicles. × 375, × 125, × 125 respectively.

FIG. 12. Section through a subcutaneous nodule comprised of gelatinous material, large polygonal cells, giant cells, blood vessels, and polymorphonuclear leucocytes. × 250.

FIG. 13. Enlargement of capillary, indicated by arrow in Fig. 12, showing involvement of endothelial cells. × 1000.

FIG. 14. Higher magnification of giant cell shown in Fig. 12. × 1000.

PLATE 36

FIG. 15. Metastasis in the lung. The epithelium of the bronchus near the myxomatous tissue is hyperplastic and contains cytoplasmic inclusions. × 150.

FIG. 16. Represents the involvement of epithelial cells in the epididymis which is characterized by hyperplasia and cytoplasmic inclusions. Compare with Fig. 22. × 300.

FIG. 17. Metastasis around blood vessel in spleen. × 125.

FIG. 18. Early lesion in lymph node. Note changes in cells lining the lymph sinus and the presence of large phagocytic cells within the sinus. × 250.

FIG. 19. Complete replacement of lymph node by myxomatous tissue. × 250.

PLATE 37

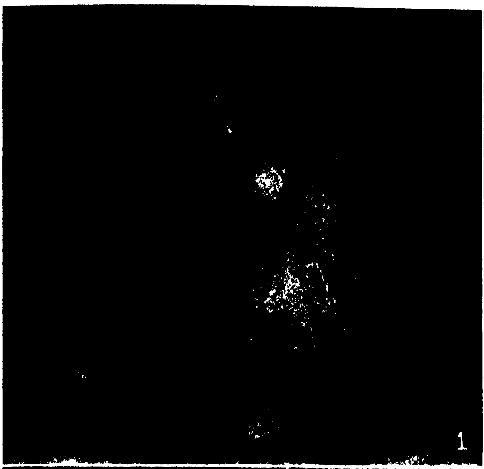
FIG. 20. Metastasis in spleen. Eosin and methylene blue. × 125.

FIG. 21. Acidophilic cytoplasmic inclusions in epidermal cells. Eosin and methylene blue. × 800.

FIG. 22. Acidophilic cytoplasmic inclusions in epithelial cells of epididymis. Compare with Fig. 16. Eosin and methylene blue. × 1000.

FIG. 23. Epidermal cell with vacuolated nucleus and granular acidophilic cytoplasmic inclusion in which are situated three blue coccoid bodies. Giemsa. × 1500.

FIGS. 24, 25. Myxoma cells. Note fragmentation of chromatin. Giemsa, and eosin and methylene blue respectively. × 1500.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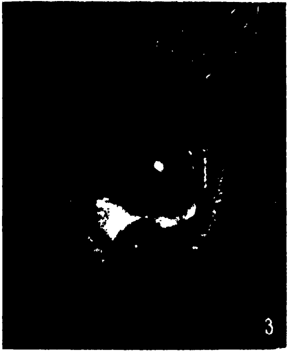
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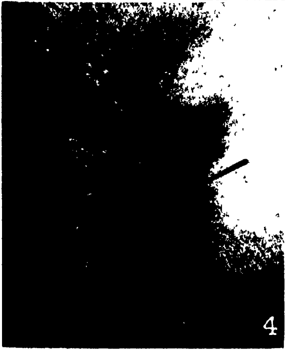
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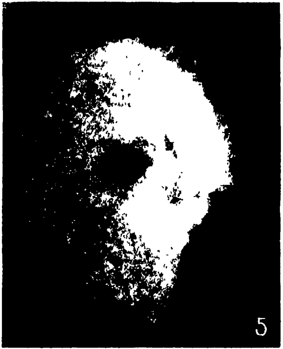
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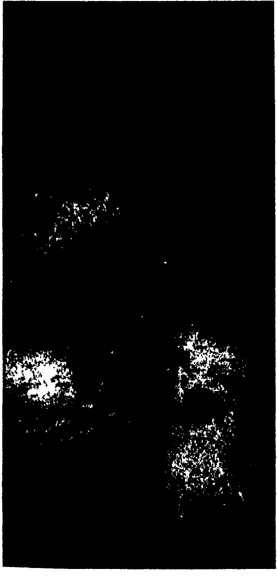

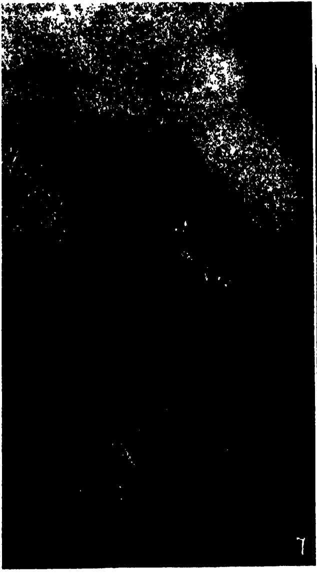
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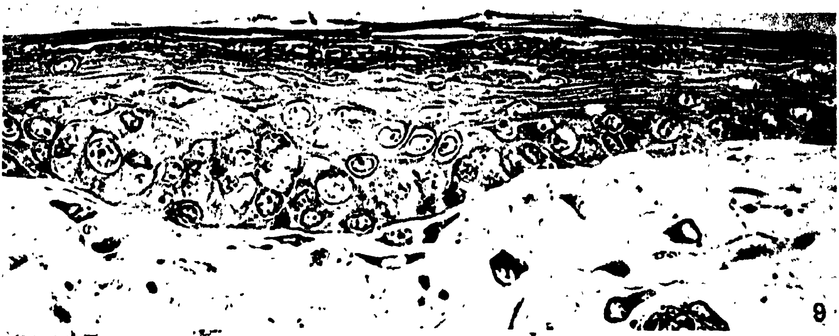
(Rivers: Infectious myxomatosis of rabbits)

PLATE 35



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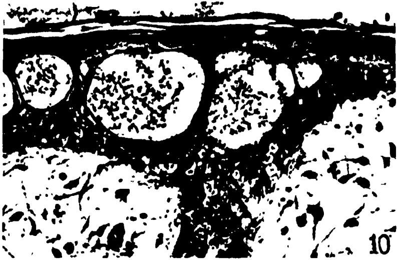

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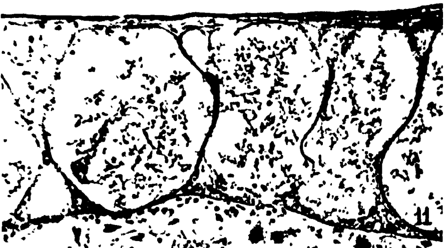

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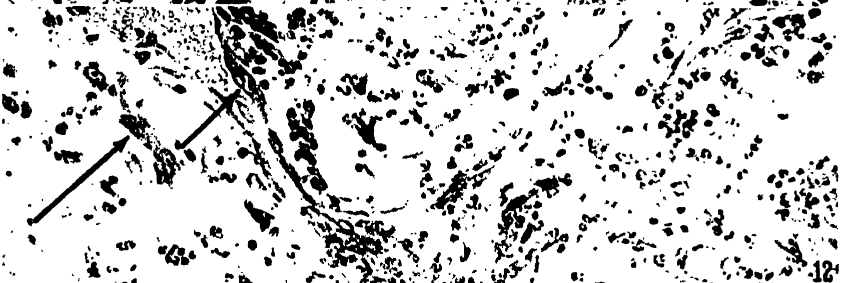

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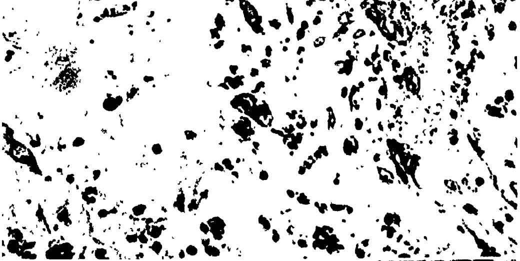
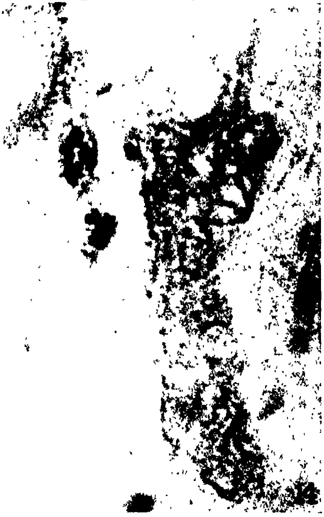
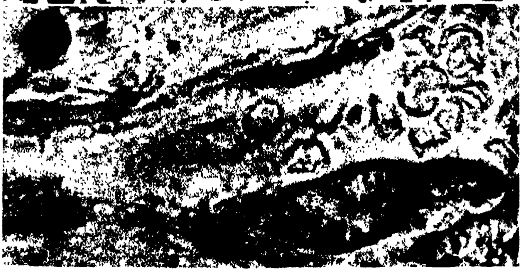
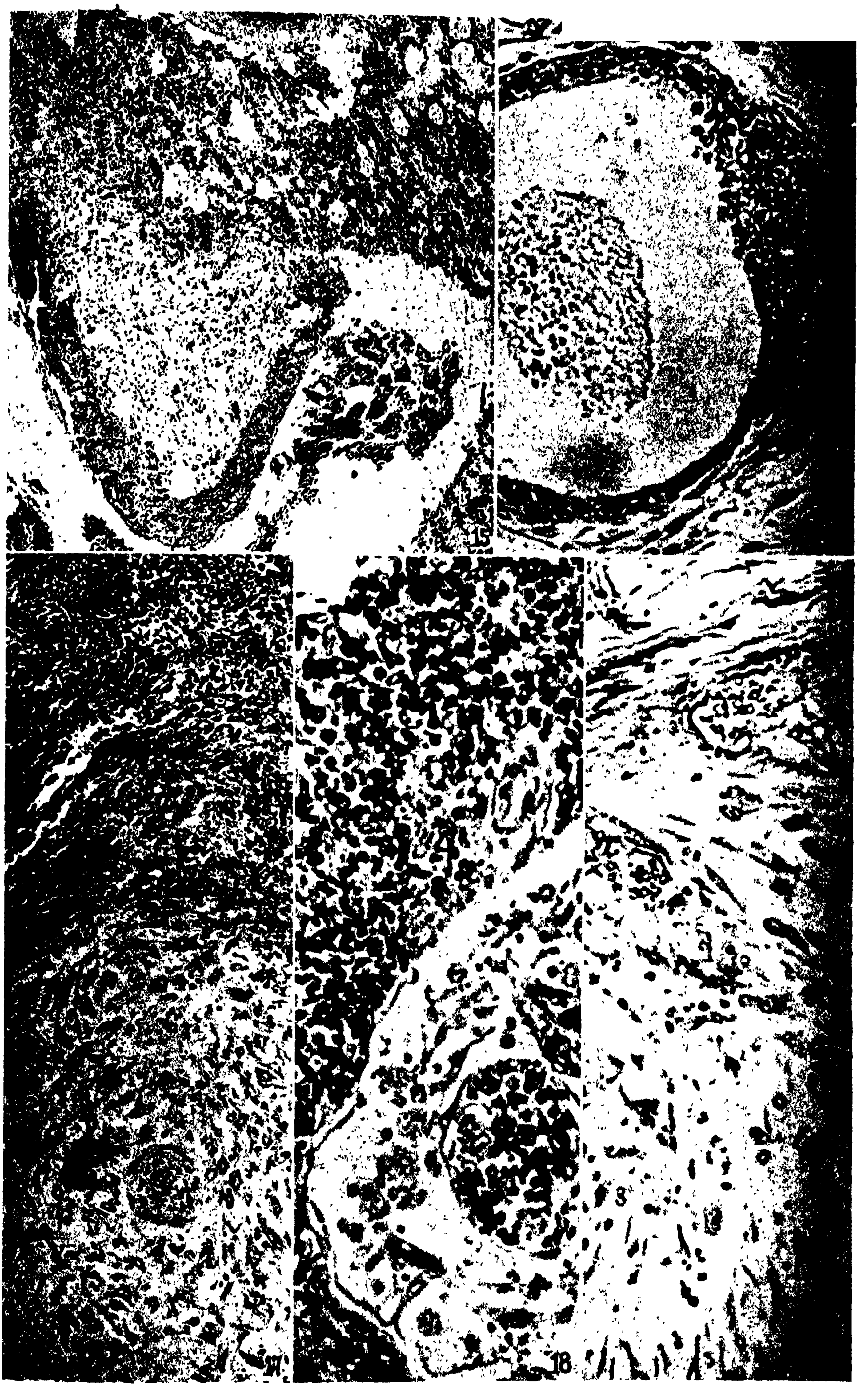






(Rivers: Infectious myxomatosis of rabbits)



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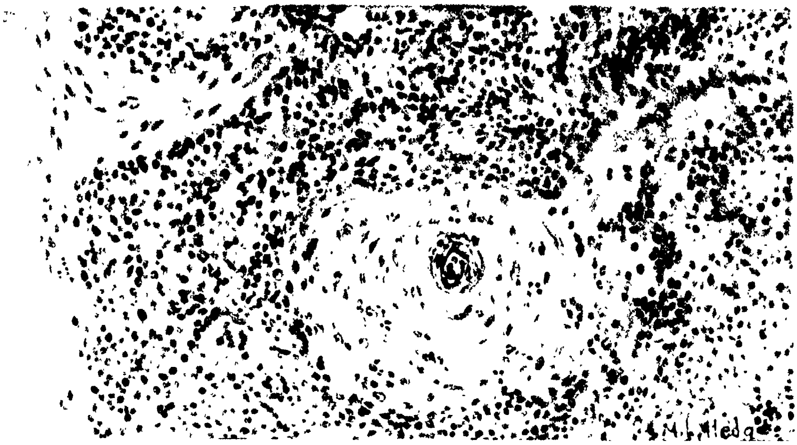

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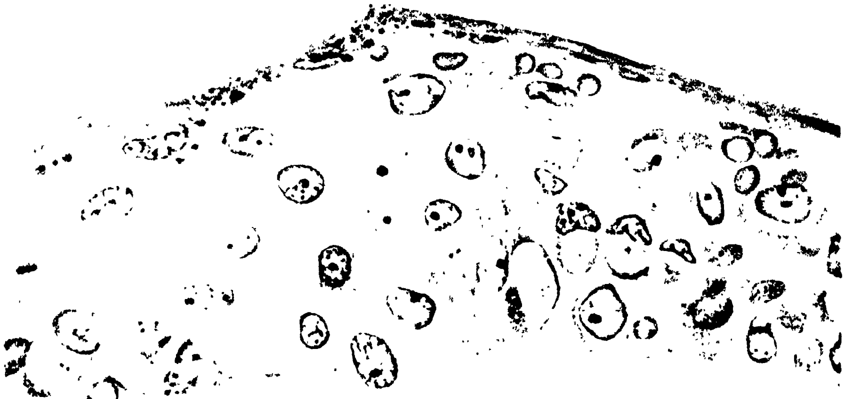

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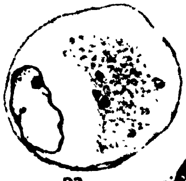

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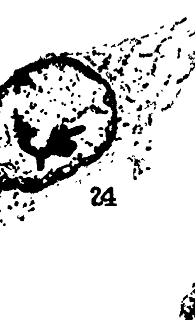

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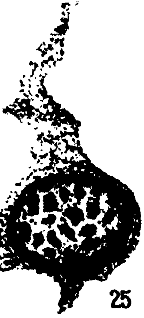

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(Rivers: Infectious myxomatosis of rabbits)

[Reprinted from the ARCHIVES OF INTERNAL MEDICINE, May, 1930, Vol. 45, No. 5, pp. 734–741]

LIPOID NEPHROSIS

REPORT OF A CASE OF UNUSUAL DURATION*

BY EATON M. MACKAY, M.D. AND CHRISTOPHER JOHNSTON, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

The literature abounds with reports of cases of chronic nephrosis; observations on the clinical manifestations of the disease, its course, its frequent termination by peritonitis and its pathology are not uncommon. We have not, however, been able to find a report of a case that has lasted so many years as the one herein discussed. According to Munk,[1] the disease may last months or years. Clausen[2] reported a case in a child, aged 13, who had had the disease since early childhood and was still living in 1924, and Lowenthal[3] reported a fatal case that lasted for nearly four years. The duration of other fatal cases has, in general, not been so long as this. In Davison and Salinger's[4] series, the longest fatal case lasted thirty-six months; one of Kaufmann and Mason's[5] patients lived thirty-one months, and one of Volhard and Fahr's[6] lived fourteen months.

Our patient was under observation for six years, and the history, which we feel is reliable, gives further evidence that the duration of the disease was at least seventeen years.

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* Through the courtesy of the Beth David Hospital and the Fifth Avenue Hospital, we were able to consult their original records of this patient.

[1] Munk, F.: Die Nephrosen, Med. Klin. 39: 41, 1916.

[2] Clausen, S. W.: Parenchymatous Nephritis, Am. J. Dis. Child. 29: 587 (May) 1925.

[3] Lowenthal, K.: Zur Frage der Lipoid Nephrose, Virchows Arch. f. path. Anat. 261: 109, 1926.

[4] Davison, W. C., and Salinger, R.: Tubular Nephritis (Nephrosis) in Children and Its Relationship to Other Forms of Nephritis, Bull. Johns Hopkins Hosp. 41: 329, 1927.

[5] Kaufmann, J., and Mason, E.: Nephrosis: A Clinical and Pathologic Study, Arch. Int. Med. 35: 561 (May) 1925.

[6] Volhard, F., and Fahr, T.: Die Brightsche Nierenkrankheit, Berlin, Julius Springer, 1914.

REPORT OF CASE

History.—M. M., a Jewish boy, was born on March 18, 1909, and died on Dec. 2, 1928. The family history is noncontributory. The patient was apparently normal at birth. He had measles at 9 months, with uneventful recovery. He gave no history of any other disease, except as noted under present illness.

Present Illness.—When he was about 16 months old, his face and cheeks became swollen, and in a few months his lower extremities started to swell. He was admitted to the Harlem Hospital for treatment for kidney disease, but was discharged unimproved. (Records of this admission are not available.) He was put on a diet in which salt, meat, eggs, pepper and spicy foods were prohibited, and was kept on this diet until he was 15 years old.

According to his mother, his urine always contained large quantities of albumin, and since the first attack he suffered from marked edema of the face, abdomen and legs at least every year, and usually oftener. The edema generally lasted but two or three weeks.

Records from the Beth David Hospital show that he was readmitted on Nov. 4, 1922 (previous records destroyed by fire) with marked edema of both legs and puffiness of the eyelids. His blood pressure was 118 systolic and 72 diastolic, his heart was normal, the lungs clear and the pharynx without injection. The patient was discharged against advice on Nov. 11, 1922.

He was readmitted on Feb. 1, 1923, with edema of the face, vomiting and resolving pneumonia of the right lower lobe. Recovery was rapid. The patient was discharged on February 11.

On July 2, 1923, his tonsils and adenoids were removed. On July 28, he was admitted with appendicitis, and at operation, pus was found in the abdominal cavity. No culture was taken. No edema was observed on admission, but on August 11, some puffiness appeared about the eyelids and soon became generalized. He apparently made a good recovery following operation and was discharged with edema on Sept. 5, 1923.

On June 1, 1925, he awakened with edema of the eyelids, and four days later was admitted to the Beth David Hospital with marked edema of the eyelids and ascites. Special examination of the optic fundi showed no changes. By July 3, there was edema of the eyelids and over the sacrum and lower extremities; marked scrotal edema had developed, and the ascites had increased. Subjectively, the patient was well. There was slight improvement after this, but he was discharged against advice on Sept. 29, 1925, with considerable edema. Various therapeutic measures had been tried. Hot packs were without effect, and there was no apparent benefit from thyroid extract in doses of from 0.05 Gm. daily, beginning June 12 and gradually increasing, to 1 Gm. daily by August 3. Daily injections of merbaphen between September 17 and 21 were without effect.

He remained at home in bed until Feb. 12, 1926, when he was admitted to the Fifth Avenue Hospital on account of massive edema of the extremities. On February 16, almost a gallon of ascitic fluid was obtained. Hot packs were tried,

TABLE I

Urinalyses in Case, from Records of Beth David and Fifth Avenue Hospitals

Date	Specific Gravity	Albumin	Casts	White Blood Cells and Epithelial Cells	Red Blood Cells	Remarks
11/ 5/22	1.025	Positive	0	0	0	These analyses were recorded during a time when the patient was markedly edematous, according to the clinical notes. It is not known whether the sediments were examined in freshly voided specimens
11/ 7/22	1.028	Positive	0	Few	0	
11/ 9/22	1.030	Positive	Granular	0	0	
11/10/22	1.025	Positive	0	0	0	
2/ 4/23	1.018	Negative	0	0	0	Clinical notes: "resolving" pneumonia; edema of face
7/30/23	1.025	Negative	0	Few	0	During peritonitis; "patient considerably edematous"
8/20/23	1.020	Trace	0	Few	0	
6/11/25 to 9/25/25	1.024 to 1.032	8 Gm./L. and +++	Few granular, few white blood cell, few hyaline	Few to moderate	None, except on Aug. 31; then occasional ones; Sept. 2, moderate	Concentration test June 17: specific gravity 1.024 to 1.034
2/12/26 to 3/18/26	.....	3 to 12 Gm./L., generally 8 to 9	Moderate hyaline, moderate granular	Few	None	Feb. 20; specific gravity 1.028 to 1.038
10/ 4/26 to 11/15/26	1.014 to 1.019	4 to 10 Gm./L., generally 8 to 9	Heavy hyaline, many finely granular	+	0	Oct. 8; specific gravity 1.013 to 1.015, which is possibly inaccurate owing to edema fluid
8/30/27 to 11/10/27	1.010 to 1.015	6 to 7 Gm./L.	Hyaline +; granular +	Few	Few noted in 2 examinations	Concentration test: specific gravity 1.021 to 1.023

but were discontinued on February 25, when he developed bronchopneumonia. Recovery was uneventful. The patient was discharged improved but with considerable edema on May 8.

TABLE 2

Data Obtained from Records of Previous Admissions to Beth David and Fifth Avenue Hospitals: Blood Chemistry and Phenolsulphonphthalein Tests

Date	Urea Nitrogen, Mg. per 100 Cc.*	Chlorides	Cholesterol*	Hemoglobin, Per Cent	Red Blood Cells, Millions	Phenolsulphonphthalein		Excretion Total
						First Hour	Second Hour	
8/25/23	....	....	306	80	8.68	..	..	..
1/23/25	....	0.57	180	..	....	..	..	40
2/ 3/25	12.6	....	...	..	....	..	..	56
6/13/25	....	....	...	75	4.32	8	12	20
6/15/25	....	....	...	..	....	12	25	37
6/18/25	25.0	0.40	220	80	....	..	..	..
7/ 1/25	12.0	....	210	..	....	..	..	..
7/10/25	....	....	...	..	....	25	18	43
7/21/25	12	0.52	...	65	4.00	..	..	..
8/ 1/25	14	0.54	...	78	4.72	..	..	..
8/ 4/25	....	....	...	..	....	35	16	51
9/ 1/25	14	0.54	...	80	5.75	30	17	47
9/ 8/25	....	....	...	78	....	32	14	46
9/10/25	12	0.52	230	..	....	..	..	..
9/21/25	....	....	...	..	....	32	20	52
2/12/26	22.9	0.57	206	70	5.0	16.6	25	41.6
2/15/26	....	....	...	86	4.8	..	..	..
3/ 4/26	9.0	0.40	...	96	5.1	..	..	..
10/ 4/26	25.0	....	...	77	3.75	..	..	..
10/13/26	18.0	....	280	60	3.21	..	..	..
11/ 2/26	....	....	...	69	3.58	15	20	35
8/31/27	14.0	....	...	34	3.1	..	..	..
9/ 1/27	....	....	...	34	3.8	15.3	6.7	22
9/ 2/27	....	....	...	..	....	20	19	39
9/ 6/27	....	....	...	30	1.92	..	..	..
10/22/27	....	....	...	55	4.00	..	..	..
10/31/27	....	....	...	58	4.10	..	..	..

* During observation at the Rockefeller Hospital the blood urea nitrogen was never above 15 mg. per 100 cc. The cholesterol varied between 167 and 510, and was generally above 300 mg. per 100 cc.

He returned to the Fifth Avenue Hospital on Oct. 4, 1926. He had not been out of bed since his discharge in May, and the swelling had varied somewhat, but was always pronounced. On admission, the blood pressure was 105 systolic and 65 diastolic. The optic fundi were pale, but otherwise normal. There was marked edema below the knees, and the abdomen was tense with fluid. He was given digitalis and thyroid extract (one tablet three times a day) throughout his stay, and was kept on a high protein, salt-free diet. On October 13, he was given a transfusion of 400 cc. of blood, and on October 29 some small tonsillar tags were removed under local anesthesia. He was discharged improved, but still considerably edematous, on Nov. 15, 1926.

The patient remained at home in bed, feeling fairly well. His appetite was fair. He had no shortness of breath. The swelling remained unaltered for the most part. He was readmitted to the Fifth Avenue Hospital on Aug. 30, 1927, on account of the edema. He was pale and poorly nourished. The lower extremities were still extremely edematous and on September 1, 7 liters of ascitic fluid was withdrawn. Liver diet and manganese were given without effect on the anemia and on October 21 the patient received a transfusion of 500 cc. of blood.

TABLE 3

Results of Tests (Addis) of Sediment of Twelve-Hour Urine

Date	Volume	Specific Gravity	Red Blood Cells	White Blood Cells and Epithelial Cells	Casts	Differential Count of Casts			
						Hyaline	Epithelial	Granular	Fatty
12/ 1/27	380	1.019	152,000	10,032,000	1,976,000	60	20	20	0
12/30/27	480	1.017	393,600	8,816,000	2,304,000	80	0	4	16
2/ 6/28	380	1.020	144,000	3,344,000	1,672,000	65	0	0	35
3/31/28	680	1.018	136,000	4,080,000	2,584,000	10	0	0	90
5/ 2/28	775	1.019	310,000	6,820,000	1,860,000	70	10	0	20
6/23/28	740	1.016	444,000	3,000,000	1,184,000	100	0	0	0
7/14/28	660	1.015	264,000	4,224,000	704,000	100	0	0	0
7/30/28	655	1.013	786,000	2,358,000	2,538,000	100	0	0	0
9/14/28	684	1.014	68,400	252,000	189,000	76	0	24	0
9/25/28	665	1.014	349,000	900,000	1,970,000	88	0	12	0
11/ 8/28	395	1.015	1,085,000	3,060,000	1,930,000	100	0	0	0

On Nov. 10, he was transferred to the Hospital of The Rockefeller Institute. According to his statement, at the time his legs were less than a third of their former size, and there had been a general decrease of the edema during the preceding six months.

Physical Examination (by Dr. E. M. Mackay).—The general appearance was that of an undernourished boy of from 10 to 12 years of age, while the actual age was 18 years. The skin was pasty and sallow. The head was normal in shape. The ears presented no inflammation. The eyes were normal. In the mouth and pharynx, the mucosa was pale, and the teeth were in poor condition. The pharynx did not show injection. The thyroid gland was not palpable. There was a marked outward flare of the lower part of the thoracic cage, probably as a result of the ascites. The diaphragm was evidently considerably elevated. No abnormal sounds were heard over the lungs. The maximum impulse of the heart was palpable in the fourth space 9 cm. to the left of the midline. Dulness extended 5 cm. to the right, and 8 cm. to the left, in the fourth space. Sounds were clear; no murmurs were heard. The radial pulses were equal and regular; the vessel walls were soft. Blood pressure was 108 systolic and 78 diastolic. The abdomen was greatly distended from ascites. Numerous striae were present. None of the organs could be palpated. The genitalia were normal. There was first degree edema of the feet and ankles. The reflexes were normal.

There was definite, though slight, impairment of the renal function, as shown on the chart, anemia, and the low plasma albumin concentration characteristic of nephrosis. Teleoroentgenographic measurements of cardiac outline showed median right 4 cm., median left 5.5 cm. The total internal diameter of the thorax was 26.5 cm. The results of a quantitative study of the sediment of the urine by the method of Addis[7] were in entire agreement with the diagnosis of nephrosis. The Wassermann test was negative.

He was placed on a low salt diet with 70 Gm. of protein daily, and there was a gradual decrease of edema as shown by the weight curve. Urea, in a dosage of from 10 to 20 Gm. daily, was given during the latter part of December, and intermittently thereafter. In February, 1928, the basal metabolism was found to be—18.

There was a gradual diminution in the amount of edema over a period of several months. In September, a general examination revealed the following features: (1) marked pallor; (2) firm, nonpitting swelling of both ankles; (3) slight clubbing of toes; (4) no enlarged lymph nodes, except at the angle of the jaw; (5) optic fundi pale, but otherwise normal; (6) few remaining teeth in good condition; (7) pharynx clear; (8) thyroid not palpable; (9) lungs clear, bases descending 4 cm. on deep inspiration; (10) relative cardiac dulness normal; heart sounds of good quality, soft systolic mormur over apex, not transmitted, and well marked sinus arrhythmia; (11) liver, soft, smooth and palpable just below the costal margin on deep inspiration; (12) no evidence of ascites; (13) spleen not palpable; (14) slight genital hypoplasia.

He was discharged on September 29, vastly improved, and remained at home with his activities much reduced, but was readmitted on Nov. 6, 1928, because of increasing edema of the legs. In addition to the edema, examination revealed occasional scattered râles in both sides of the chest. The liver edge was palpable on deep inspiration, and the spleen was indefinitely felt on deep palpation.

At 8 p. m. on November 27, the patient had a chill, followed by a febrile rise to 105.6. Examination disclosed only slight tenderness along the left side of the abdomen. By the next morning, the abdominal pain had become generalized, and the temperature remained at 105.4. A diagnosis of general peritonitis was made. Operation was not performed, but on December 1 abdominal paracentesis yielded a thin, milky-looking fluid in which were many gram-positive cocci. Culture disclosed a pure culture of hemolytic streptococci.

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[7] Addis, T.: The Number of Formed Elements in the Urinary Sediment of Normal Individuals, J. Clin. Investigation 2: 409, 1926.

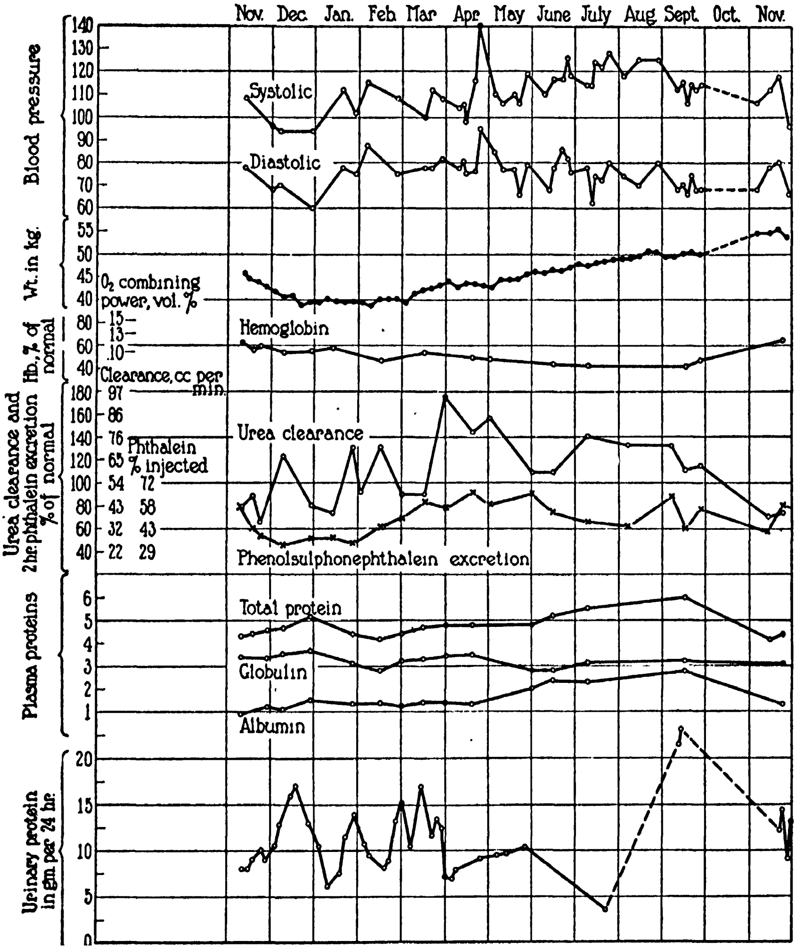
The patient died on December 2, on the seventh day of the acute illness.

COMMENT

It is unfortunate that the early records of this case cannot be traced. Nevertheless, the history as given here agrees in its essentials with histories obtained from the patient's mother at both the Beth David and the Fifth Avenue Hospitals, except that on one occasion the age of onset was given as 4 years. The story of recurring periods of edema and of the constant (?) presence of albuminuria is common to all the records, and in view of the course of the disease, the absence of hypertension, the later clinical picture and the observations at autopsy, it would be difficult to imagine that the earlier attacks were due to glomerulonephritis. Furthermore, the records of the examinations of the urine show that albuminuria was constant, except at two observations, both of which may be doubted because the clinical notes state clearly that edema was present. Rare red blood cells were said to have been seen on only four occasions in the many examinations made. The urine was not examined for doubly refractile lipoid bodies prior to November, 1927, but they were found present in large numbers on every examination thereafter.

The treatment of this patient is not without interest. From the onset of the disease until March, 1926, when he was 15 years old, he was on a low protein diet. The change to high protein diet at this time was without demonstrable effect, but as we do not know how much the protein intake was restricted, nor how much it was later increased, conclusions are not justified. During the last year of the patient's life, the edema decreased while he was on a high protein diet (120 Gm. daily), but urea was given successfully as a diuretic at the same time, and the plasma albumin did not increase until the daily loss of protein in the urine had decreased somewhat. Thyroid extract was tried repeatedly and in doses up to 1 Gm. daily without effect. Merbaphen, theobromine sodium salicylate, digitalis, calcium chloride, pituitary extract and urea were tried repeatedly. On one occasion, a diuresis started coincident with digitalis medication, and the good effect of urea and of calcium chloride on the urine output was definite. Other medication was entirely without effect.

Foci of infection were sought for, and the records showed that the tonsils and adenoids were removed in July, 1923, October, 1926, and



Graphic record of the weight and the results in examinations of the blood and the urine of a patient with lipoid nephrosis.

April, 1928. No focus was ever found in the paranasal sinuses. The teeth were badly decayed and were removed one by one until few remained at the time of death. The patient recovered completely from an attack of pneumonia in 1923, and bronchopneumonia in

1925. At an operation for appendicitis in 1923, pus was found in the abdominal cavity. There was no apparent relationship between any of these infections and the course of the disease.

The general development of the patient, it has already been noted, was not commensurate with the actual age. The general bodily conformation and the secondary sex characteristics were those of a boy of from 12 to 14 years of age. This is not remarkable in view of the fact that for almost the first fifteen years of life his diet must have been greatly deficient in protein, and the developmental delay cannot in this case be considered as a specific effect of the disease, as is thought to be the case in examples of so-called renal dwarfism. Furthermore, x-ray pictures showed that in our patient, the skeletal development was normal for his age.

Although anemia is not ordinarily encountered in nephrosis, reference to the tables shows that our patient had a secondary anemia which was at first only slight, but which gradually became more severe. It is especially to be noted that although the erythrocytes occasionally fell to low levels, there was, in general, no indication of deficient erythropoiesis in spite of the two factors of disease and prolonged restricted diet during the period of growth. During his stay in the Rockefeller Hospital, the hemoglobin showed a steady decline in spite of a high protein diet including eggs, and the additional administration from March 5 to May 19, 1928, of six vials of liver extract daily, representing 600 Gm. of fresh liver. The red count increased from 4,030,000 to 4,800,000 while the patient was receiving liver extract, but in September, four months after its discontinuance, and at a time when the hemoglobin values were lowest, the red count was found to vary from 4,950,000 to 5,600,000. These latter determinations were checked by two observers, using different pipets and counting chambers and taking blood from either the finger or the ear. The increase in hemoglobin between September and November, 1928, is attributed to the use of pills of ferrous carbonate, U. S. P. (0.3 Gm. three times daily) from September 14 to 28 and from November 6 to 28, and was accompanied clinically by a definite improvement in the patient's color.

There has been much discussion, especially in the German literature, over the question of the secondarily contracted kidney of nephrosis

(Munk;[8] Lowenthal;[3] Volhard and Fahr[6]), and it has apparently been established that such cases do occur. We wish merely to call attention to the fact that in this case of such long duration, there was no clinical evidence of secondary contraction of the kidney, as there was no accompanying hypertension, cardiac enlargement or retention of nitrogen.

It is likely that a great many cases reported as "pure nephrosis" are in reality cases of glomerulonephritis accompanied by great tubular degeneration, edema, low plasma proteins and the other signs of nephrosis, and distinguished from pure nephrosis only by the presence of slight hematuria. Addis[7] showed that normal persons may excrete as many as 425,000 erythrocytes in the urine in twelve hours, and did not consider any count as significant of glomerulonephritis unless the excretion is above 1,000,000. In eleven such counts of the sediment of the urine in this case, none of them gave any evidence that there was any glomerular lesion. Furthermore, the phenolsulphonphthalein tests showed no great reduction in function, and what is perhaps of even greater significance, the test for blood urea clearance showed that this was never greatly depressed below the low normal value of 80 per cent, and was for most of the time well above normal.

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[8] Munk, F.: Zur Pathogenesis der nephrotischen Schrumpfniere, Virchows Arch. f. path. Anat. **226:** 81, 1919.

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Vol. 87, No. 2, pp. 265–279]

STUDIES OF GAS AND ELECTROLYTE EQUILIBRIA IN BLOOD

XVI. THE EVOLUTION OF CARBON DIOXIDE FROM BLOOD AND BUFFER SOLUTIONS

BY DONALD D. VAN SLYKE AND JAMES A. HAWKINS

(From the Hospital of The Rockefeller Institute for Medical Research)

(Received for publication, April 7, 1930)

Henriques (1928), using Faurholt's data (1924) on the velocity of hydration of CO_2 and the dehydration of H_2CO_3 and HCO_3^- at 0° and 18°, calculated the velocity of dehydration of H_2CO_3 at 38° and pH 7.40. He assumed that under average conditions, in the change from venous to arterial blood in the lung, 11.75 volumes per cent of bound CO_2 must be freed from the erythrocytes. He calculated from Faurholt's data that by change of HCO_3^- and H_2CO_3 into anhydrous CO_2 only 1.95 volumes per cent could be freed. He concluded from this that a large part of the carbon dioxide in the erythrocytes must be in the form of anhydrous CO_2 loosely bound to hemoglobin, "carbhemoglobin." As evidence in confirmation of this hypothesis he showed that during a time when from serum exposed to a vacuum only 22 volumes per cent CO_2 were set free, from whole blood 28 volumes per cent were set free.

Henriques has also explained, by assuming the existence of "carbhemoglobin," the fact that the CO_2 content of the erythrocytes is higher than it would be if the Donnan equation

$$\frac{[HCO_3]_c}{[HCO_3]_s} = \frac{[Cl]_c}{[Cl]_s} = \frac{[H^+]_s}{[H^+]_c}$$

held with activity coefficients of unity for all of the ions concerned. (The subscripts *c* and *s* are used to indicate concentrations in cells and serum respectively.) The fact that the HCO_3 ratio, calculated on the assumption that all the bound CO_2 is in the form of bicarbonate, is higher than the Cl ratio was shown by Warburg (1922) and by Van Slyke, Hastings, Murray, and Sendroy (1925). The latter authors demonstrated that the HCO_3 ratio thus calculated is, in terms of molar concentrations, 25 per cent higher than the Cl ratio, and assumed that the difference was due to the effect of unknown factors on the activity coefficients of the two types of ions. Both the Cl and the HCO_3 ratios were found higher than the ratios of H^+ activities determined by the hydrogen electrode. Henriques stretches his theory to explain also the fact that the Cl ratio is higher than the H^+ activity ratio; he assumes that not only part of the CO_2, but a part, somewhat less, of the Cl in the cells is also bound to the hemoglobin.

Regardless of the mechanism attributed to it and the hypotheses based on it, the behavior of hemoglobin in accelerating the evolution of CO_2 from blood is a phenomenon of sufficient physiological importance to deserve confirmation.

Henriques' experimental data do not appear in themselves sufficient to demonstrate such an acceleration. He showed that when whole blood was agitated in an evacuated chamber CO_2 escaped from the fluid more rapidly than from serum similarly treated. Such a difference could, in part at least, be attributed to the well known acidic and buffer properties of hemoglobin, which retard the rise of pH when CO_2 escapes from whole blood, and thereby make it possible completely to free the latter of CO_2 by agitation in an evacuated space; while serum exposed to a vacuum becomes so alkaline (from the change $NaHCO_3 \rightarrow Na_2CO_3$) that only about half of the CO_2 can be removed. For comparison with blood, it appears essential to use a solution similar, not only in initial CO_2 content and pH, but also in buffer value, so that throughout the evolution of CO_2 the pH of the compared solution shall approximate that of whole blood or hemoglobin solution which has lost similar proportions of its CO_2.

We have therefore prepared solutions of bicarbonate, carbonic acid, and phosphates which meet these requirements. When agitated under reduced pressure they do, in fact, evolve CO_2 at a much slower rate than whole blood or solutions of hemoglobin. The results thoroughly confirm Henriques' conclusion that hemoglobin accelerates the escape of CO_2 gas.

That the acceleration is due to the existence of quantitatively important proportions of CO_2 bound in the form of "carbhemoglobin," however, appears to us improbable for reasons that will be given in the discussion of the results.

EXPERIMENTAL

Equilibration of Blood, Serum, and Solutions with Carbon Dioxide

The blood serum or solutions were saturated at 38° with 40 mm. of CO_2, the double tonometers described by Austin *et al.* (1922) being used. 100 cc. of solution were saturated with 1000 cc. of gas. The lower chamber of the tonometer was filled with solution and clamped off from the upper except during the period of saturation. After 1 hour's saturation, the gas mixture was renewed twice and two ½ hour saturations were made in order to make certain that the final conditions should approximate as closely as possible those desired.

The CO_2 tensions were determined, as described in connection with the saturation method, Austin *et al.* (1922), by analysis, by Haldane's method, of the separated gases in the upper chamber of the tonometer.

The total CO_2 contents of the solutions were determined by means of the manometric apparatus of Van Slyke and Neill (1924), the factors of Van Slyke and Sendroy (1927) being used to calculate the amount of CO_2.

The hemoglobin concentration was determined by the colorimetric method of Palmer (1918).

The amount of protein in the serum was determined by the gasometric micro-Kjeldahl method of Van Slyke (1927).

Symbols Used

The following symbols are used:

$[BHCO_3]$, $[H_2CO_3]$, $[CO_2]$ represent concentration in mM per liter of $NaHCO_3$, physically dissolved CO_2, and total CO_2 respectively in the solutions.

β represents the molecular buffer value for the buffers in solution other than sodium bicarbonate.

The carbon dioxide tensions in mm. of mercury are expressed as p_{CO_2}.

α is the Bunsen solubility coefficient of CO_2.

(Hb) represents concentration of hemoglobin in mM per kilo of blood.

Constants

The values of pK′, used in the equation $\text{pH} = \text{pK}' + \log \frac{[\text{BHCO}_3]}{[\text{H}_2\text{CO}_3]}$ were 6.15, 6.10, and 6.17 for blood, serum, and 0.08 M phosphate solution, respectively. The value of pK′ for whole blood was taken from the nomogram of Van Slyke, Hastings, Murray, and Sendroy (1925). The value of pK′ for serum was taken from Hastings, Sendroy, and Van Slyke (1928). The value of pK′ for bicarbonate in the phosphate mixtures was calculated from the equation $\text{pK}' = 6.33 - 0.5 \sqrt{C}$, where C, the calculated concentration in mols per liter, is substituted for μ, the ionic strength used by Hastings and Sendroy (1925) for bicarbonate and sodium chloride mixtures, when the effect of the phosphates, mol for mol, is assumed to be the same as that of sodium chloride and bicarbonate.

The solubility coefficient for CO_2, α_{CO_2}, was 0.485, 0.511, and 0.528 for blood, serum, and 0.08 M phosphates, respectively (Van Slyke, Sendroy, Hastings, and Neill (1928)). The value of α_{CO_2} for the phosphate mixtures was obtained by subtracting the appropriate value for the decrease in solubility of CO_2 due to the concentration of the phosphate and bicarbonate; *i.e.*, $0.08 \times 0.2 = 0.016$ and $0.02 \times 0.13 = 0.002$ from $\alpha_{CO_2} = 0.546$ for water.

The value of $\text{pK}' = 6.79$ for the phosphate solution used in the equation $C = \frac{\beta}{0.575U}$ was taken from Cohn (1927).

Calculations

The concentration of $[H_2CO_3]$ was estimated from the formula $[H_2CO_3] = 0.591\ \alpha_{CO_2} \times p_{CO_2}$. The concentration of $[BHCO_3]$ was determined by subtracting $[H_2CO_3]$ from $[CO_2]$. The calculation of pH from $[BHCO_3]$ and $[H_2CO_3]$ was made by use of the Henderson-Hasselbalch equation (Van Slyke, Sendroy, Hastings, and Neill (1928)).

The buffer value of serum was calculated as 0.104 × gm. of serum protein per liter, the albumin:globulin ratio being assumed to be 1.6 (Van Slyke, Hastings, Hiller, and Sendroy (1928)).

The concentration of hemoglobin necessary to give a desired buffer value was calculated by means of Equations 52 and 17 (Van Slyke, Wu, and McLean (1923)), $\beta = 3.6 \text{ (Hb)} + 0.068 \text{ (P)}_s$, where $\text{(P)}_s = 0.072 - 0.0039 \text{ (Hb)}$.

The buffer value of blood was calculated from the hemoglobin content by the above equation.

The concentration in mols per liter of phosphate of given pH required to give a desired buffer value was calculated as $C = \frac{\beta}{0.575U}$ by the use of Fig. 9 in the paper of Van Slyke on buffers (1922). The U curve there shows, for a pH point at any given distance either side of the pK′ of the given buffer, the percentage of maximal buffer value that will be exerted at that pH. The maximum buffer value of any buffer solution is $0.575\ C$ and is exerted when pH = pK′, C being the molar concentration of the buffer, in this case phosphate. Hence the buffer value of any phosphate is $0.575\,CU$, where U is the factor from Fig. 9.

Evolution of CO_2 from Blood, Serum, and Buffer Solutions under Reduced Pressure at Various Time Intervals

A solution with the required pH and double the concentration of phosphate necessary to give the calculated buffer value of blood, and a solution with double the required amount of sodium bicarbonate necessary to give the same $[NaHCO_3] : [H_2CO_3]$ ratio at 40 mm. tension of CO_2 as blood were made up. The two solutions were then mixed, giving a solution with the same pH, buffer value, and $[NaHCO_3] : [H_2CO_3]$ ratio as that of blood.

Undiluted ox blood and the above buffer solution were used. The red cells of the blood were laked with 0.1 per cent saponin. Formation of lactic acid was prevented by the addition of 0.1 per cent sodium fluoride. The saponin and sodium fluoride were added to the phosphate solution as well as to the blood so that conditions in both solutions would be the same. The blood and phosphate solutions were then equilibrated at 38° with CO_2 at 40 mm. tension of mercury.

The amount of CO_2 evolved at various time intervals from blood and buffer solutions was determined by means of the Harington-Van Slyke constant volume apparatus (1924).

The apparatus was washed once with N H_2SO_4 and then three times with distilled water to insure that it was free of acid. The apparatus was evacuated and shaken. The mercury was then run back into the apparatus and any air expelled through the upper stop-cock. 1 drop of octyl alcohol was introduced into the apparatus. The upper stop-cock was sealed with mercury and the apparatus evacuated till the mercury was just above the lower stop-cock. The lower stop-cock was then closed.

The blood or solution was then transferred quantitatively by means of the 5 cc. pipette, described by Van Slyke and Neill in Section XII of their paper (1924). One arm of the Y-shaped stop-cock at the top of the pipette was joined by means of a short rubber tube to the side arm on the lower stop-cock of the Harington-Van Slyke extraction chamber. Mercury was expelled from the lower chamber of the Harington-Van Slyke apparatus through the Y arm of the Ostwald pipette to remove any air. The Y stop-cock was turned and the solution was run into the lower bulb of the gas apparatus, followed by mercury to seal the stop-cock of the gas apparatus. Care was taken that the blood or solution was run into the apparatus without being placed under reduced pressure at any time. The pipette was then detached from the gas apparatus.

The solution was allowed to run into the upper chamber of the gas apparatus along with sufficient mercury so that the meniscus of the mercury was at the 50 cc. mark. The apparatus was at once shaken vigorously for the required time interval. The solution was then drawn down into the lower chamber, leaving the gas in the upper chamber. The time required to admit the solution to the upper chamber of the Harington-Van Slyke apparatus and drain it out was 10 seconds.

The solution was expelled from the lower chamber of the apparatus through the side arm on the lower stop-cock along with enough mercury to seal the stop-cock.

5 cc. of acidified distilled water were admitted to the upper chamber from the cup at the top and shaken in the evacuated chamber to establish equilibrium with the CO_2 there. The lower stop-cock of the extraction chamber was opened and mercury was admitted until the water meniscus was exactly at the 2.0 cc. mark; then a manometer reading, p_1, was taken.

TABLE I

Evolution of CO_2 from Ox Blood and Solutions with Same Buffer Value, pH, CO_2 Tension, and CO_2 Content as That of Blood

Solution.	$p\mathrm{CO}_2$	$[\mathrm{H_2CO_3}]$	Total $[\mathrm{CO_2}]$.	$[\mathrm{BHCO_3}]$	$\frac{[\mathrm{BHCO_3}]}{[\mathrm{H_2CO_3}]}$	pH	Hb concentration in terms of O_2 capacity.	β	Temperature.	CO_2 evolved during various time intervals. Time of shaking, sec.									
										5	10	20	40	80	5	10	20	40	80
										mM per liter.					Per cent of total CO_2.				
	mm.	*mM*	*mM*	*mM*			*vol. per cent*		*°C.*										
Ox blood.	41.0	1.19	20.68	19.49	16.4	7.37	20.1	30.71	23.5	3.87	3.96	4.18	4.39	4.44	19.98	20.28	21.40	22.48	22.73
	41.5	1.20	19.54	18.35	15.4	7.34	21.7	33.04	23.0	3.26	3.39	3.73	4.02	4.38	16.70	17.36	19.10	20.58	22.42
	41.6	1.20	22.28	21.08	17.6	7.39	20.0	30.58	23.0	3.12	3.50	4.16	4.40	4.38	15.40	15.70	18.90	19.75	19.67
Buffer 0.08 M phosphate and bicarbonate.	40.0	1.25	20.38	19.13	15.3	7.36		30.59	24.0	1.69	1.80	2.16	2.78	3.89	8.29	8.83	10.61	13.65	19.11
	40.6	1.26	19.98	18.72	14.9	7.34		30.59	23.5	1.64	1.78	2.10	2.74	3.73	8.20	8.91	10.51	13.72	18.66
Buffer 0.08 M phosphate, bicarbonate, and ox blood.	40.0	1.23	20.85	19.62	16.0	7.37	1.1	30.59	25.0	4.72	5.36	5.69	6.12	6.26	22.62	25.70	27.30	29.35	30.00
	41.2	1.28	20.80	19.52	15.3	7.35	1.0	30.59	24.0	4.92	5.39	5.59	6.10	6.26	23.66	25.91	26.88	29.32	30.10

3 drops of 5 N NaOH were introduced through the upper stop-cock to absorb the CO$_2$. After the stop-cock was again sealed with mercury, the meniscus of the water was brought to the 2.0 cc. mark and a second reading, p_2, taken.

The CO$_2$ evolved from the blood or solution was then determined by multiplying $p_2 - p_1$ by the factors calculated from the formula of Van Slyke and Sendroy (1927) with the constants $a = 2.0$, $S = 5.0$, and sample = 5.0 cc.

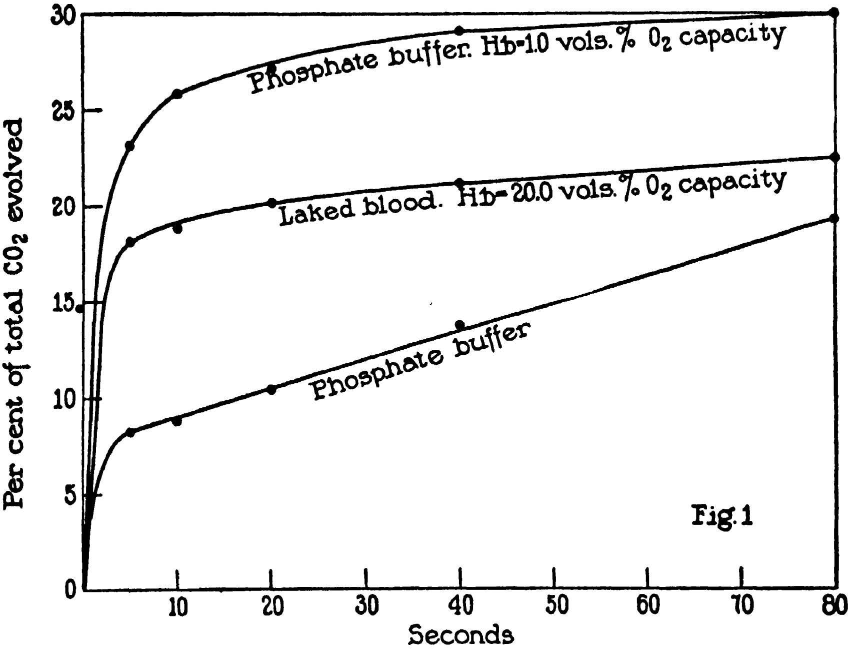

FIG. 1. Comparison of the rate of liberation of CO$_2$ from whole ox blood, phosphate buffers with the same buffer value as that of blood, and from similar phosphate buffer containing red blood cells. Ordinates represent per cent of total CO$_2$ liberated; abscissæ represent seconds of shaking in the Harington-Van Slyke apparatus.

The results of these experiments are shown in Table I and Fig. 1. It is seen that as much CO$_2$ is evolved from the blood in 10 seconds as from the phosphate solution in 80 seconds. The evolution of CO$_2$ from the phosphate solution proceeds at a steady ratio after the first 5 seconds, 40 per cent of the total CO$_2$ being liberated during the first 5 seconds. The evolution of CO$_2$ from laked blood

TABLE II

Evolution of CO_2 from Ox Serum and Solutions with Same Buffer Value, pH, CO_2 Tension, and CO_2 Content as That of Serum

Solution.	pCO_2	$[H_2CO_3]$	Total $[CO_2]$.	$[BHCO_3]$	$\frac{[BHCO_3]}{[H_2CO_3]}$	pH	Hb concentration in terms of O_2 capacity.	β	Temperature.	CO₂ evolved during various time intervals. Time of shaking, sec.									
										5	10	20	40	80	5	10	20	40	80
										mM per liter.					Per cent of total CO_2.				
	mm.	*mM*	*mM*	*mM*			*vol. per cent*		°C.										
Ox serum.	42.0	1.27	26.20	24.93	19.7	7.39		7.78	23.0	1.26	1.36	1.78	2.32	2.89	4.81	5.19	6.79	8.86	11.04
	41.3	1.25	25.51	24.26	19.4	7.38		7.90	25.0	1.34	1.47	1.93	2.50	3.13	5.30	5.76	7.56	9.80	12.27
Ox blood laked, 0.1 M sodium chloride, and bicarbonate.	42.0		24.62				4.8	7.34	23.5	3.00	3.23	3.54	3.55	3.57	11.98	13.15	14.43	14.46	14.54
	43.0		27.04				4.7	7.20	23.0	2.94	3.17	3.76	3.96	3.98	10.88	11.74	13.92	14.67	14.72
Ox serum.	38.7	1.17	27.58	26.41	22.6	7.45	0.3	6.98	24.0	2.11	2.50	2.77	3.25	3.42	7.66	9.06	10.05	11.78	12.40
	41.0	1.24	30.25	29.01	23.4	7.47	0.3	7.08	25.0	2.15	2.34	2.72	3.44	3.73	7.11	7.74	9.00	11.37	12.33
Ox blood unlaked, 0.1 M sodium chloride and bicarbonate.	42.0		24.62				4.8	7.34	23.5	1.92	2.24	2.69	3.11	3.36	7.82	9.12	10.96	12.66	13.68
	43.3		24.71				4.7	7.20	23.0	1.99	2.53	2.83	3.24	3.58	8.03	10.24	11.44	13.12	14.48

is very rapid during the first 5 seconds as 80 per cent of the CO_2 freed is liberated during this interval. This agrees with the finding of Henriques ((1928) p. 5) that CO_2 is evolved more rapidly when hemoglobin is present in solution.

In order to determine whether a solution free of hemoglobin, but containing other proteins in sufficient concentration to give a similar buffer value, would behave differently, similar experiments were performed with undiluted ox serum.

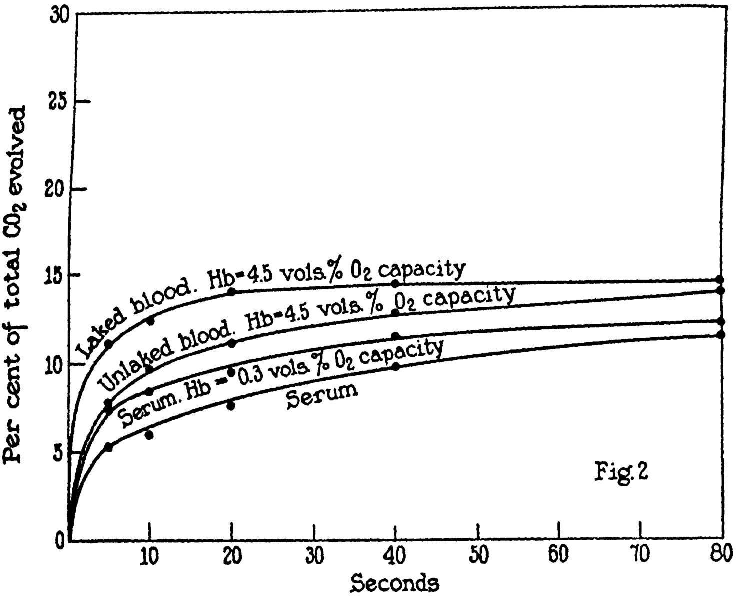

FIG. 2. Comparison of the rate of liberation of CO_2 from serum and from blood diluted so that its buffer value is the same as that of serum. Ordinates represent per cent of total CO_2 liberated. Abscissæ represent seconds of shaking in the Harington-Van Slyke apparatus.

Undiluted ox serum and ox blood, diluted with solution containing 100 mM of sodium chloride and 25 mM of sodium bicarbonate to give the same buffer value as that of serum, were used. The red cells of the blood were laked with 0.1 per cent saponin. Formation of lactic acid was prevented by the addition of 0.1 per cent sodium fluoride. The saponin and sodium fluoride were added to serum as well as to the blood solution so that conditions would be the same in both. In a second series of experiments the saponin was not added so that the red cells were unlaked. The serum and blood mixtures were then equilibrated at 38° with CO_2 at 40 mm. tension of mercury.

The results of these experiments are shown in Table II and Fig. 2. It is seen that as much CO_2 is evolved from the diluted laked blood solution in 5 seconds as from serum in 80 seconds.

The evolution of CO_2 from serum proceeds at a steady rate between 5 and 40 seconds and then falls off slowly. The evolution of CO_2 from the diluted blood with the same buffer value and at the same tension as the serum is very rapid during the first 5 seconds; 73 per cent of the CO_2 liberated is freed during this interval. After 20 seconds shaking, no more CO_2 escapes from this solution.

This finding again agrees with those of Henriques in attributing to hemoglobin a unique ability to accelerate the evolution of CO_2 from its solution.

The diluted unlaked blood, the serum of which contained 0.3 per cent hemoglobin, and the serum containing 0.3 per cent hemoglobin gave curves which lay between those of the laked blood solution and serum.

To ascertain the relation between hemoglobin concentration and the accelerating effect of hemoglobin on CO_2 evolution, a relatively slight proportion of red cells was added to the bicarbonate-phosphate buffer solution previously described.

6 cc. of ox blood were added to 120 cc. of the buffer of 0.08 M phosphate and 0.02 M bicarbonate containing 0.1 per cent saponin and sodium fluoride to make the hemoglobin concentration about 5 per cent that of whole blood. The mixture was saturated at 38° with CO_2 at 40 mm. tension. Portions of the solution were then agitated in the evacuated Harington-Van Slyke chamber, and the amounts of CO_2 evolved in various time intervals were determined.

It is seen from Table I and Fig. 1 that the addition to the buffer solution of 0.05 the concentration of red cells that exists in blood increased the rate of liberation of CO_2 from solution so that it was comparable with the rate of liberation of CO_2 from the whole blood. A larger amount of CO_2 was evolved from the blood-buffer solution than from the blood, but the ratio of initial evolution speed to amount of gas finally liberated was approximately the same, 77 per cent of the finally liberated amount of CO_2 escaping in the first 5 seconds.

DISCUSSION

Henriques' conclusion, that the presence of hemoglobin accelerates the evolution of CO_2 from blood, is entirely confirmed by the above results.

However, his hypothesis that the acceleration is due to the binding of a great part of the CO_2 in the cells in the form, not of bicarbonate, but of anhydrous CO_2 gas loosely united to hemoglobin, does not appear to be consistent with our finding that 0.05 the concentration of hemoglobin present in blood causes, in a solution of similar pH and buffer power, an approximately equal acceleration of CO_2 evolution.

Furthermore, Henriques' assumption that an important proportion of the bound CO_2 is not in the form of bicarbonate is inconsistent with quantitative data published in previous papers of this series, the significance of which Henriques appears to have overlooked. The amounts of alkali bound by hemoglobin at varying pH levels have been determined independently by two methods. (1) Crystals of purified hemoglobin have been dissolved in known amounts of standard NaOH or KOH solution, which was then saturated with CO_2 at known tensions, the quantitative relationships of hemoglobin, alkali, pH, and CO_2 being so chosen that they resembled those occurring in blood *in vivo* (Van Slyke, Hastings, Heidelberger, and Neill (1922); Hastings, Van Slyke, Neill, Heidelberger, and Harington (1924)). It was assumed in calculating the results of these experiments that the alkali not bound to hemoglobin was combined with CO_2 in the form of bicarbonate. The base, BHb, bound to hemoglobin was therefore calculated as follows:

$$\text{BHb} = (\text{total alkali added}) - \text{BHCO}_3$$

the bicarbonate being calculated as

$$\text{BHCO}_3 = (\text{total CO}_2) - \text{H}_2\text{CO}_3$$

The symbol, H_2CO_3, is used to represent all the free dissolved carbonic acid, both that actually in the form of H_2CO_3 and in the form of anhydrous CO_2. The amount of such free carbonic acid was calculated from the determined tension of CO_2 gas and the solubility coefficient of CO_2 in water solutions of the nature employed.

It is obvious from the formulæ of calculation that if important amounts of CO_2, in excess of those calculated from the solubility of the gas in water solutions, had remained in a form other than bicarbonate, a gross error would have been caused in the calculated value of $BHCO_3$, and consequently of BHb.

(2) However, Hastings, Sendroy, Murray, and Heidelberger (1924) found the same proportions of alkali combining with hemoglobin at the same pH levels by an entirely different procedure, carried out without CO_2. They dissolved hemoglobin in measured amounts of NaOH solution and titrated back with HCl, determining the pH after each HCl addition by means of Hastings' rotating hydrogen electrode. For reduced hemoglobin and for carbon monoxide hemoglobin (which has the same alkali-binding power as oxyhemoglobin) the same proportions of alkali bound per mol of hemoglobin at the same pH were found, within the limits of experimental error, as by the CO_2 titration method, outlined above.

It therefore appears that, if the accuracy of these results is accepted, one must either (1) accept the assumption that even in the presence of hemoglobin practically all of the CO_2 is divided between the forms of free carbonic acid and bicarbonate, or else (2) one must assume that, even on the alkaline side of the isoelectric point, free HCl forms with hemoglobin the same sort of indefinite compound that Henriques has assumed CO_2 to form, and in the same proportions at the same pH levels. The latter assumption is, we believe, too far outside the limits of probability to merit consideration. The fact that titration of alkali hemoglobinate with HCl and with CO_2 both indicate the same alkali-combining power of hemoglobin constitutes, in the belief of the writers, evidence against the existence of quantitatively important proportions of CO_2 in any form such as that assumed for "carbhemoglobin" by Henriques.

SUMMARY

The rates of evolution of CO_2 from blood and phosphate buffers have been determined. As much CO_2 is liberated from blood or erythrocyte solution in 5 seconds as from a phosphate or serum protein solution with the same CO_2 content and buffer value in 80 seconds. These findings agree with those of Henriques.

The addition of red cells to the phosphate buffer in sufficient quantity so that the hemoglobin concentration is 0.05 that in blood accelerates the liberation of CO_2 so that the rate is as rapid as from whole blood.

We have at present no explanation of the mechanism by which the cell contents (probably but not certainly hemoglobin) accelerate CO_2 evolution from solution.

The direct combination of anhydrous CO_2 with hemoglobin assumed by Henriques is without proof of existence. That the carbon dioxide in the blood is accounted for by the amounts present as bicarbonate and physically dissolved carbon dioxide has, furthermore, been shown by quantitative data published from this laboratory.

The rapidity of evolution of CO_2 from evacuated blood solutions appears to be best described as due to a catalytic acceleration by the cell contents of the reaction $HCO_3^- + H^+ \rightleftharpoons H_2CO_3$. Concerning the mechanism of the catalysis no data are at present available for the formation of a reasonably probable hypothesis.

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LIPOID NEPHROSIS OF UNUSUAL DURATION

II. PATHOLOGIC-ANATOMIC REPORT

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It is my object to present in this paper the pathologic-anatomic changes in a case of lipoid nephrosis which furnishes a valuable contribution not only to the question of the terminal stage of this disease but also to other questions. When speaking of lipoid "nephrosis," I am fully conscious of the inadequacy of this term. Aschoff[1] was right to deduce that this term is incorrect and should be replaced by lipoid "nephrodystrophia" or "nephropathia." If here, nevertheless, the term "nephrosis" is used, it is done not only because it is generally accepted by the clinicians, but especially because it is not proved whether this disease is of a purely degenerative nature, as is supposed by Volhard and Fahr[2] and Munk,[3] or whether it is an inflammation to begin with, as is believed by Aschoff,[4] Schlayer[5] and Bohnenkamp.[6]

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5. Schlayer: Ueber die Nephrosen, Med. Klin. 14: 53, 1918.

6. Bohnenkamp, H.: Zur Frage der Nephrosen, Virchows Arch. f. path. Anat. 236: 380, 1922.

Aschoff supported his opinion mainly by the fact that in all slides of genuine nephrosis presented to him by Volhard, he found changes in the glomeruli which indicated older inflammatory processes. Furthermore, he ascribed to the epithelial cells defensive properties which he believed to be the essence of inflammation. Bohnenkamp, who followed similar ideas, relied mainly on the Uranium experiments of Suzuki.[7]

The number of cases reported in which the patients have come to autopsy is still small and may not exceed from twenty to thirty. In all, the duration was from a few months to three years. In my case the condition lasted for about seventeen years, and the patient was under continuous clinical control during the last six years. Before discussing the case, I shall report the observations. As the clinical part has been reported in detail by Johnston and MacKay,[8] I shall call attention only to the important points of the clinical observations.

REPORT OF CASE

History.—M. M., a man, aged 19, had had attacks of edema of from two to three weeks' duration at least every year, and usually more often, since the sixteenth month of life. In January, 1923, he had pneumonia, in July of the same year, appendicitis with "pus in the abdominal cavity," and in February, 1926, bronchopneumonia. During the last six years, there were recurring periods of edema and oliguria. The amount of albumin was 1.4 per cent. In the sediment casts, fatty epithelial cells, leukocytes and many doubly refractile lipoid bodies, were observed. Rare erythrocytes were said to have been seen, but on only four of the many examinations made. No cardiac enlargement was noted, no retention of nitrogen and no increase in the blood pressure. There were no symptoms of syphilis. On Nov. 27, 1928, the patient developed symptoms of diffuse peritonitis and died on Dec. 2, 1928.

Autopsy.—Only a partial autopsy was permitted. This was performed about two hours after death. The body was that of a fairly well nourished man, showing no rigor mortis but a slight livor mortis. The body showed marked edema of the legs and face. The peritoneal cavity was opened through a small incision and was found filled with about 2 quarts of greenish, purulent fluid (*Streptococcus hemolyticus*) peritonitis.

The kidneys were large and pale; each weighed about 300 Gm. The capsule stripped easily. The surface was pale yellowish and was smooth, with indications of fine, light violet depressions. Cross-section showed the cortex much enlarged,

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[7] Suzuki, T.: Zur Morphologie der Nierensekretion, Jena, 1912.

[8] Johnston, C., and MacKay, E. M.: Lipoid Nephrosis; Report of a Case of Unusual Duration, Arch. Int. Med., this issue, p. 734.

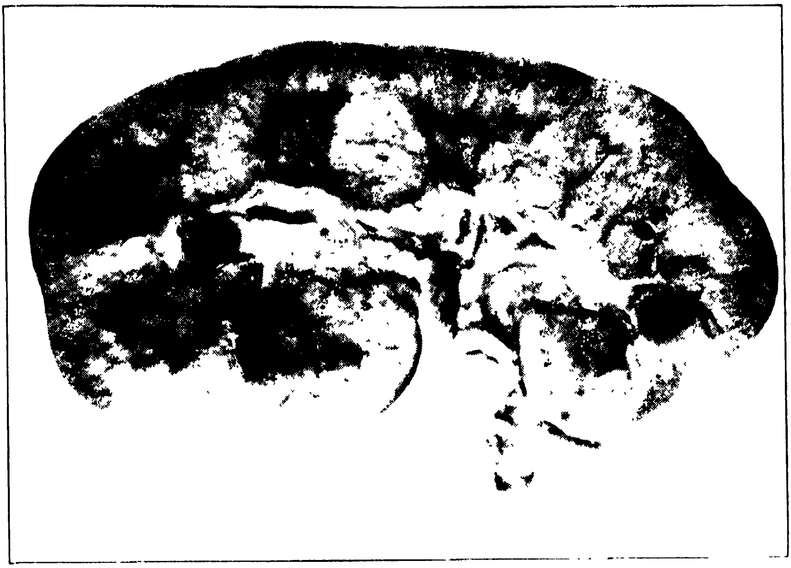

FIG. 1.—Longitudinal section through the right kidney after Jores fixation

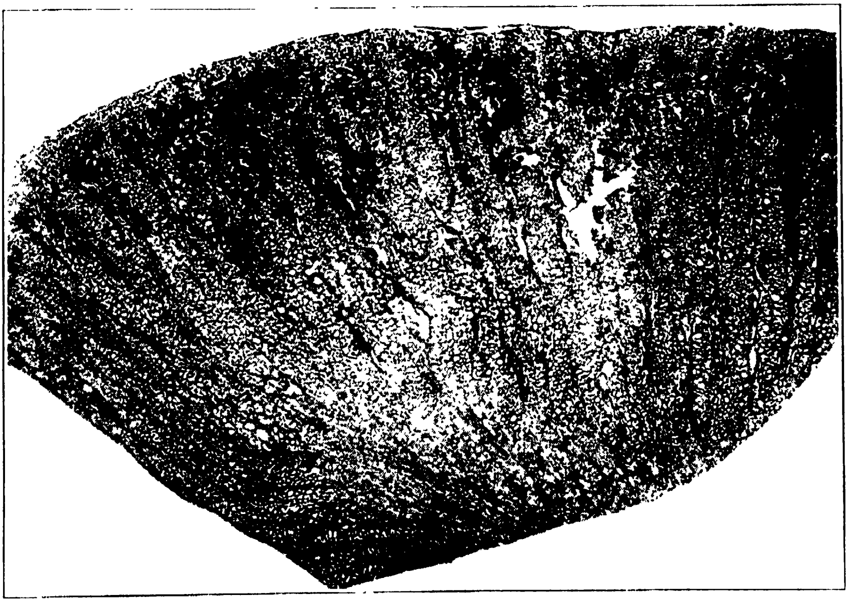

FIG. 2.—Low magnification showing the grossly striated structure of the cortex. Iron-hematoxylin-eosin; ×5.

measuring from 9 to 20 mm. It was pale yellowish and contained fine, light violet striae radiating inward from the capsule (fig. 1). The cortex was fairly well marked off from the medulla. In the pelvis a few hemorrhages were seen.

Microscopic Examination.—The normally more uniform cortical structure had, in most places, been replaced by broad striations radiating inward from the capsule to the medulla (fig. 2). A narrow, light, subcapsular zone was joined by an irregular zone which was similar to the usual cortical structure. From here, broad, light colored areas, separated by narrow, dark striae, converged toward the medulla.

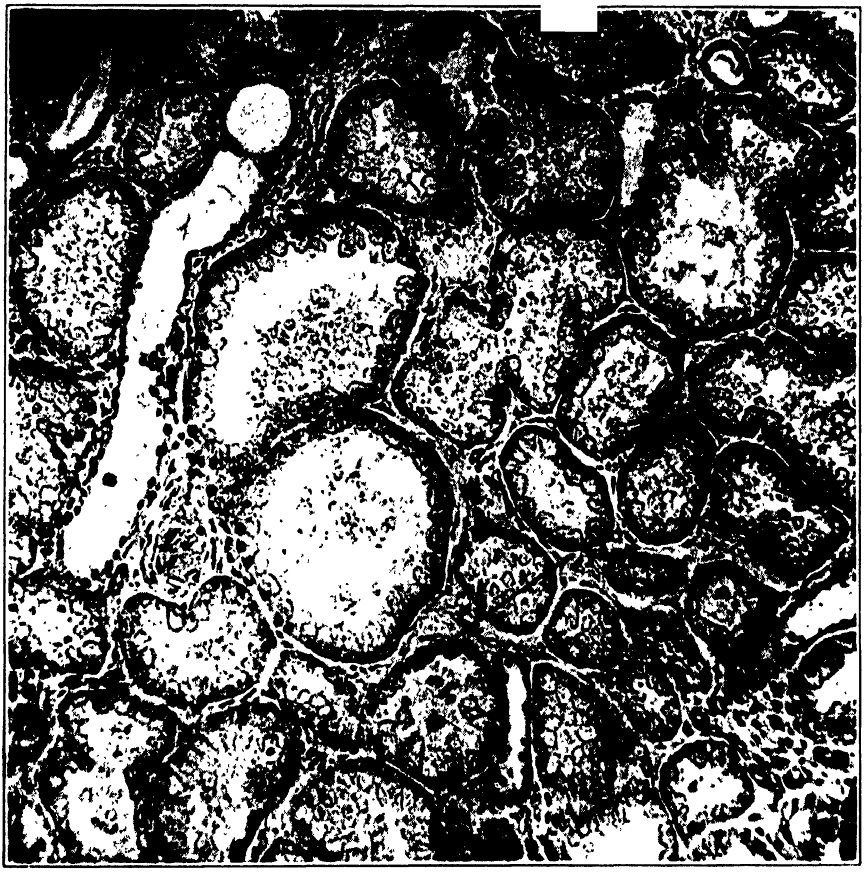

FIG. 3.—Partly hyaline droplet-like, partly fatty or vacuolated degenerated major pieces (convoluted tubules). In the lumina of the tubules is mainly coagulated protein. Regeneration in a gap of the epithelial layer (*a*). The interstitial tissue is well preserved. Iron-hematoxylin-eosin; ×160.

Under higher magnifications, the lighter parts were seen to correspond mainly to enlarged tubules, the character of which as major pieces[9] was still distinct in many places (fig. 3). These tubules were lined partly by greatly swollen and vacuolated or fatty degenerated epithelial cells which in some instances entirely closed the tubules, and partly by epithelial cells which had undergone considerable hyaline droplet-like degeneration (fig. 4). For the most part, these vacuolated and hyaline droplet-like degenerated tubules lay alternating side by side. In other places they had a more localized distribution. In some instances they passed over into each other. It should be mentioned that the hyaline droplet-like degenerated

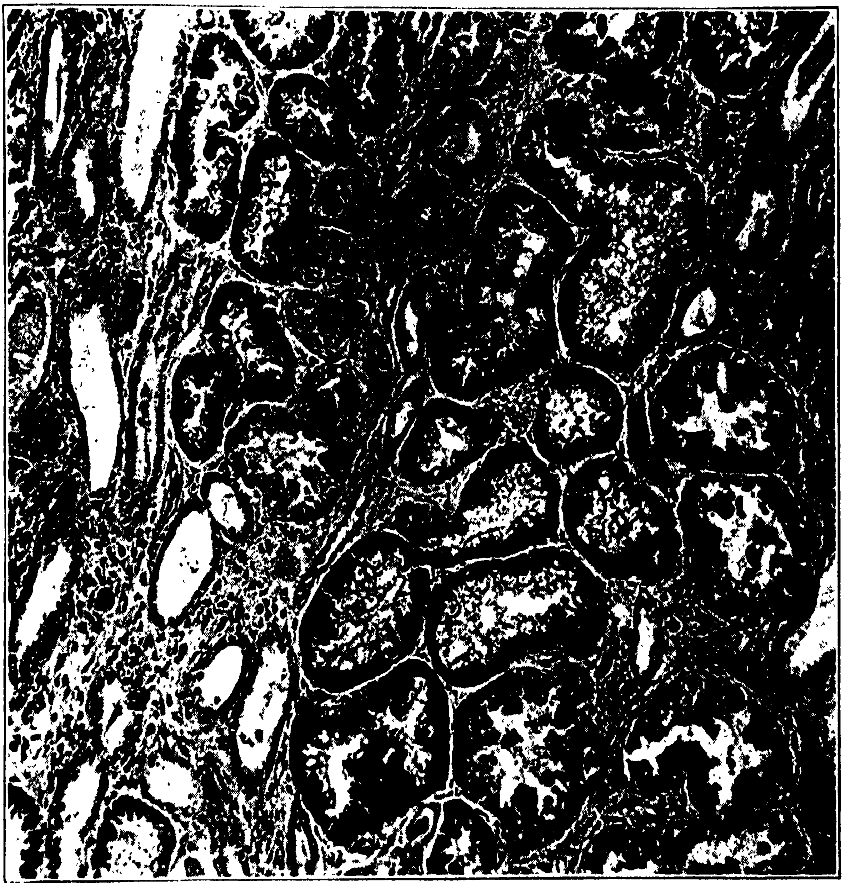

FIG. 4.—Extensive hyaline droplet-like degeneration of major pieces. Gram stain; ×160.

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[9] The term "major pieces" refers to the convoluted tubules lined by large epithelial cells in contradistinction to the tubules lined by small epithelial cells, which are designated as minor parts.

tubules were located in the half of the cortex next to the medulla. The epithelial cells, mainly of the hyaline droplet-like degenerated major pieces, frequently contained pyknotic nuclei or had lost them entirely. In many places the epithelial cells were desquamated. In such places one found signs of regeneration. Gaps in the epithelial layer were covered by epithelial cells which resembled endothelium (fig. 3) and contained karyokinetic figures here and there. In the basal parts of the epithelial cells one saw nearly everywhere fine fat globules, most of which were doubly refractile. Within the lumina of the tubules one found coagulated protein, some desquamated epithelial cells, and in some places also polymorphonuclear leukocytes. In the hyaline droplet-like degenerated major pieces one saw also hyaline drops within the lumina. The minor parts of the light parts of the cortex contained fairly large numbers of casts, which increased in number toward the medulla. The interstitial tissue of these parts of the cortex appeared to be normal in most places (fig. 3).

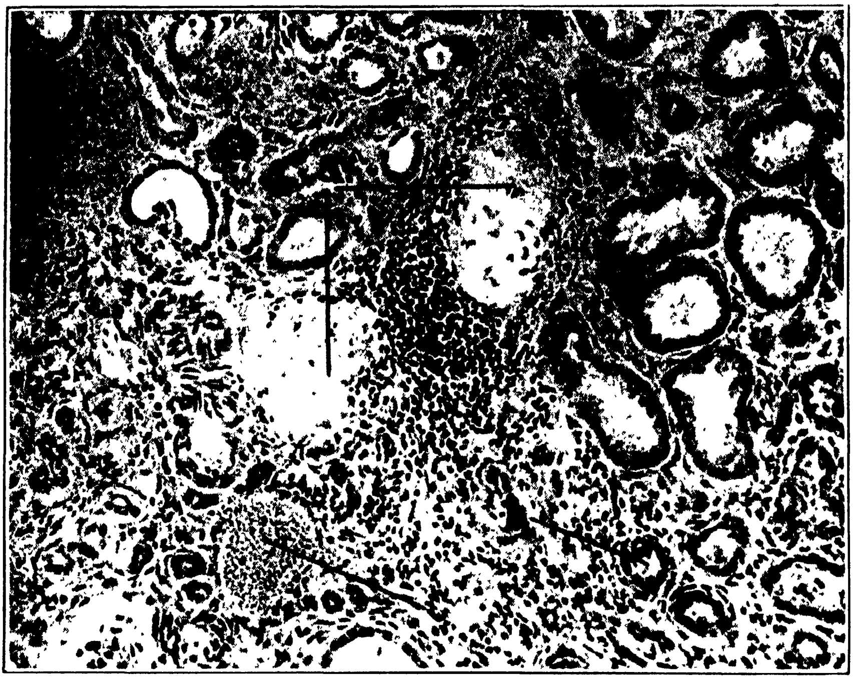

FIG. 5.—Scar with two completely hyalinized glomeruli (*a*). Between them is an infiltration of small, round cells. Degenerated tubule with a few surrounding polymorphonuclear leukocytes (*b*) is seen and (*c*) a small vein dilated with blood. Iron-hematoxylin-eosin; ×160.

Whereas the histology of the light parts of the cortex was fairly uniform, that of the dark parts was more irregular. Here the interstitial tissue was frequently increased in amount and contained increased collagenous connective tissue. One saw occurring infiltrations of small, round cells which in part adjoined hyalinized glomeruli (fig. 5). In other places, the interstitial tissue was swollen and contained polymorphonuclear leukocytes, many of which were seen entering the tubules. Everywhere in the interstitial tissue of the dark parts of the cortex one often found large, xanthoma-like cells (fig. 6), which were stained yellowish by sudan and which were doubly refractile. The tubules of the scarred parts were in all stages of atrophy and degeneration up to entire disappearance. Their epithelial cells were not, or only very little, enlarged, and were much more desquamated than those of the light part of the cortex. Many of them contained a large amount of fat, which was irregularly arranged. Occasionally one saw signs of regeneration. In some places one found calcified casts.

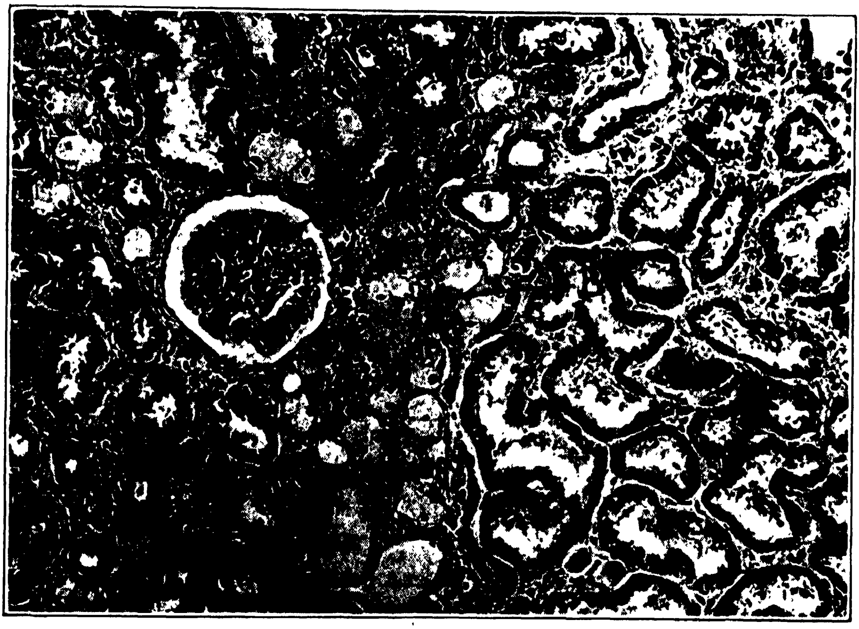

FIG. 6.—Intact glomerulus well filled with blood (*a*). In the interstitial tissue many xanthoma-like fat cells (*b*) are shown. Iron-hematoxylin-eosin; ×160.

The number of glomeruli appeared to be much decreased, but this decrease seemed to be only relative, caused mainly by the enlargement of the kidneys. Most of the glomeruli were located in the dark parts of the cortex. Fifty per cent of the glomeruli—500 were differentiated—were well preserved (fig. 6), some being of normal size and some enlarged. The enlarged glomeruli contained, according to the method of Mertz,[10] increased numbers of nuclei, but the increase corresponded

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[10] Mertz, A.: Ueber die quantitativen Zellverhältnisse der Glomeruli bei Glomerulonephritis, Centralbl. f. allg. Pathol. u. path. Anat. **29:** 321 (1918).

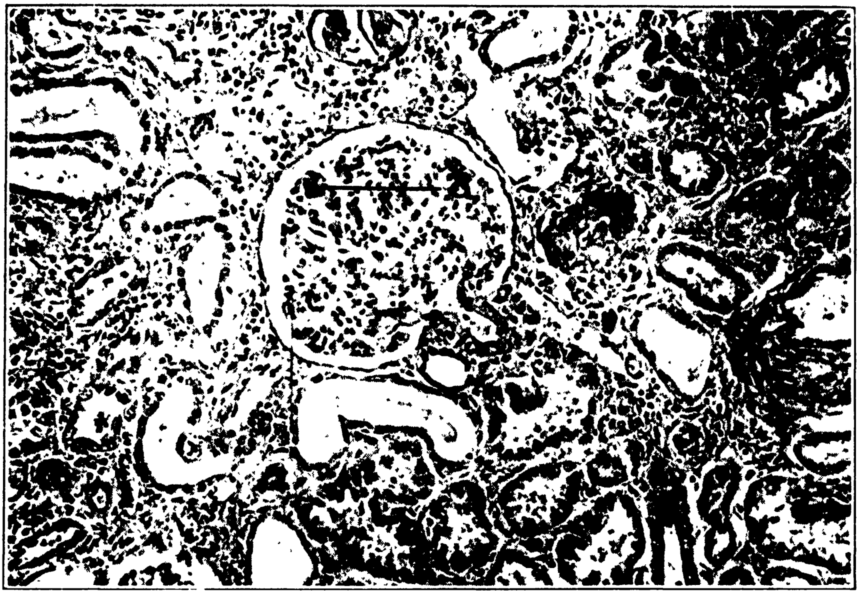

FIG. 7.—Enlarged glomerulus with a small accumulation of endothelial cells in a single loop (*a*). Elastica-van Gieson stain; ×160.

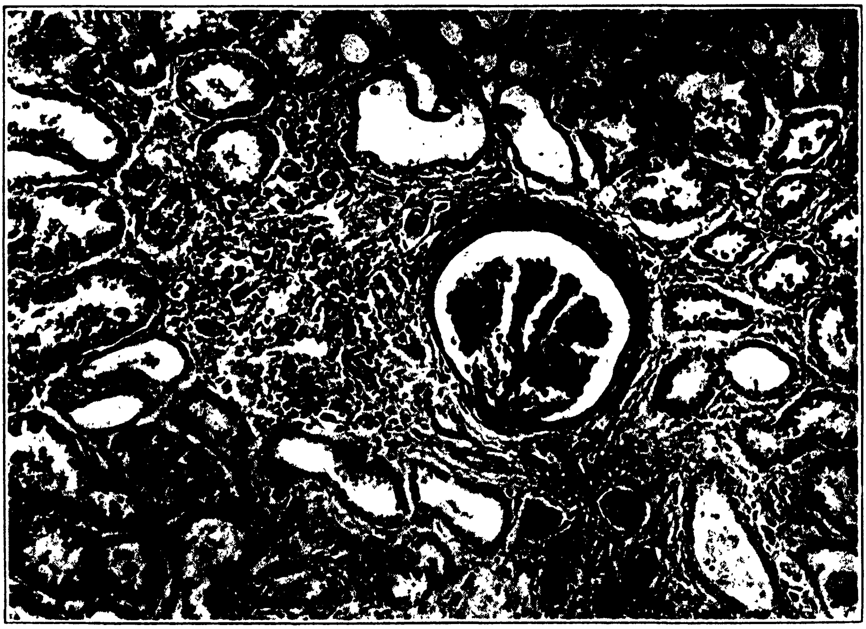

FIG. 8.—Concentric thickening of a glomerular capsule, poor in nuclei, with beginning hyalinization of the glomerulus at the place of entry of the artery. In the surrounding interstitial tissue there is an increased number of collagenous fibers (appearing dark in the photomicrograph). Elastica-van Gieson stain; ×160.

to the increase in the size of the glomeruli. In a few places one also found small accumulations of endothelial cells in a single loop (fig. 7). The loops of the well preserved glomeruli were delicate and mostly well filled with blood. Occasionally, the glomerular spaces were dilated and contained coagulated protein. There was only little fat to be found in the covering cells of the glomeruli. In these glomeruli, inflation or adhesions of the loops, increased numbers of polymorphonuclear leukocytes or protoplasmic masses within the loops were nowhere to be found. There were no erythrocytes and no fibrin in any of the glomerular spaces. No hernia-like descension of loops into the tubules was seen, nor were there adhesions of the glomeruli to the capsules. Neither were the epithelial cells of these glomeruli changed, except for the slight fatty degeneration already mentioned.

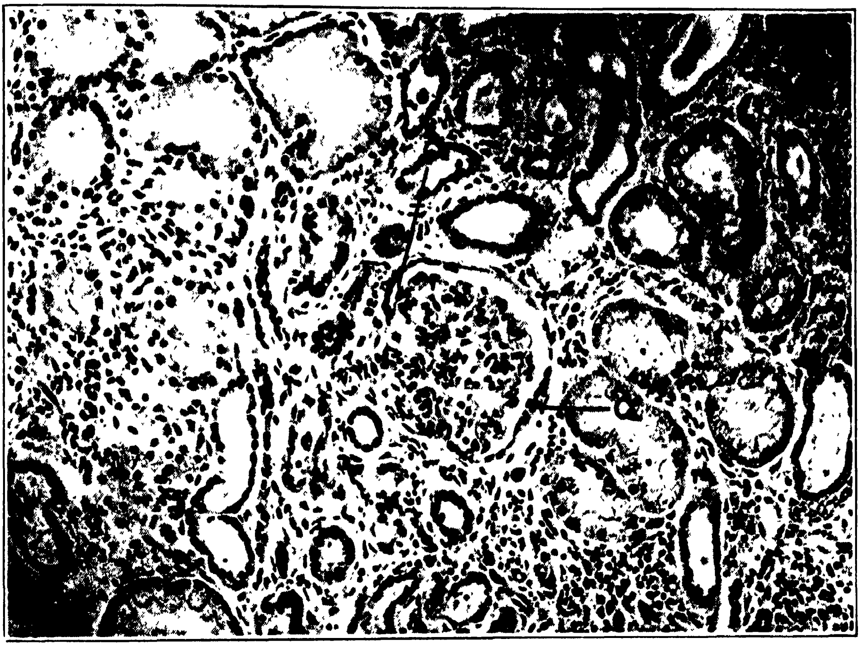

FIG. 9.—Advanced hyalinization of a glomerulus with a slight proliferation of the parietal epithelial cells (*a*). Iron-hematoxylin-eosin; ×160.

The other 50 per cent of the glomeruli were in all stages of hyaline degeneration. They were localized in the scarred parts of the cortex, partly single and partly in small groups. One saw well preserved glomeruli, the capsules of which were concentrically thickened by a substance which stained red by van Gieson (fig. 8). Other glomeruli contained hyalinized loops, especially at the place of entry of the vessels (fig. 9). These glomeruli were occasionally adherent to the capsule. A few of these glomeruli showed a slight proliferation of the parietal capsular epithelium (fig. 9), but there were no crescent formations. About a third of the glomeruli were entirely hyalinized (fig. 5) and were sharply demarcated from the surrounding tissues.

The medulla was rich in collagenous connective tissue, which was swollen in places and contained some polymorphonuclear leukocytes and round cells. In the tubules of the medulla many casts were seen and in one place also a few erythrocytes. The arterioles were well preserved. The larger arteries showed a moderate degree of intimal hyperplasia.

All amyloid reactions made were negative.

COMMENT

In a discussion of this case one has to consider first whether or not one is dealing with a genuine lipoid nephrosis. Under the differential diagnostic point of view, there has to be considered only the subchronic glomerulonephritis of Volhard,[11] with nephrotic tendency, i.e., the intracapillary form of Fahr,[12] Munk[13] especially called attention to the difficulties which frequently arise in differentiating late stages of "subacute glomerulonephritis" from nephrosis, anatomically. Both of them show the picture of large white kidney. Volhard,[14] however, mentioned that in a subchronic glomerulonephritis one usually sees macroscopic hemorrhagic dots on the renal surface. Fahr[15] even stated that small hemorrhages are invariably present. Such dots were entirely absent in the case herein reported.

Microscopically, in subchronic glomerulonephritis, the glomerular lesions are outstanding. One finds enlargement and anemia of the glomeruli, enormous increase in number of the endothelial elements, adhesions of the loops and erythrocytes in the glomerular spaces. In this case, none of these changes were found. Most of the well preserved glomeruli seemed to be entirely normal. It is true that a few glomeruli showed increased numbers of nuclei and that, in single hyalinized glomeruli, slight proliferations of the parietal epithelial cells were seen, but these observations are in entire accord with the observations of Fahr[16] and others, who stated that one finds them more frequently in later stages of lipoid nephrosis. The increase in the number of nuclei is, as Fahr believed, of a secondary inflammatory nature. If one takes into consideration that in this case the increase was not out of proportion to the size of the glomeruli, and that no other inflammatory lesions were found, it is reasonable to assume that here the increase of nuclei was merely an expression of compensatory hyperplasia of these glomeruli. Unfortunately, little is known about the histology of compensatory hypertrophy and hyperplasia of the glomeruli, and it seems to be necessary to study this question thoroughly before it can be discussed further. Concerning the proliferation of the parietal epithelial cells, which was observed in single hyalinized glomeruli, one may join Volhard,[14] who considered it a plain reaction of the epithelial cells to the hyalinization.

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[11] Volhard (footnote 2, first, second and third references).

[12] Fahr (footnote 2, first and seventh references).

[13] Munk (footnote 3, third reference).

[14] Volhard (footnote 2, second and third references).

[15] Fahr (footnote 2, seventh reference).

[16] Fahr (footnote 2, fourth, fifth and sixth references).

Furthermore, if the clinical observations are taken into consideration—I mention again only the absence of increase in blood pressure which is emphasized so much by Volhard[14]—there can be no more doubt that in this case there was a genuine lipoid nephrosis. The occasional reports of the presence of erythrocytes in the urine cannot change the diagnosis, mainly because they are said to have been seen on only four of the many examinations made, and the accuracy of some of these examinations has already been questioned.[8] Slight hemorrhages have been seen also by Volhard and Fahr[17] and other observers. Fahr[18] occasionally found them within the tubules. According to him, they are small hemorrhages from overcrowded capillaries caused by passive congestion. In the case herein reported, likewise, some erythrocytes were found, but in one tubule only, and there were also hemorrhages in the pelvis of the kidneys which may account for the occasional presence of erythrocytes in the urine.

It is well known that the question whether or not there is a genuine nephrotic contracted kidney is much disputed. Whereas Munk[19] and Fahr[18] stated the belief that the lipoid nephrosis leads to contracted kidneys, Aschoff,[4] Volhard[14] and Herxheimer[20] expressed great doubts

17. Volhard and Fahr (footnote 2, first reference).

18. Fahr (footnote 2, fourth and fifth references).

19. Munk (footnote 3, second and third references).

20. Herxheimer: Ueber den jetzigen Stand unserer anatomischen Kenntnisse der Nephritis und Nephropathien, Munchen. med. Wchnschr. 65: 283, 1918.

concerning this theory. The two cases presented in the monograph of Volhard and Fahr[17] and also the case of Mason[21] cannot stand severe criticism. In spite of a duration of seventeen years, the patient in the case herein reported still had much enlarged kidneys. Although as a rule one case is of no great importance, one may be entitled to draw conclusions in this case, especially because it seems to be the only genuine case of lipoid nephrosis of long duration ever observed. It seems to be certain that lipoid nephrosis can last indefinitely, in some cases at least, without leading to contracted kidneys.

The cause of death in the case reported was peritonitis, so common in lipoid nephrosis. The organism, however, was not a pneumococcus, as is usually the case, but *Streptococcus hemolyticus*.

SUMMARY

A case of genuine lipoid nephrosis has been reported in which the conditions lasted for about seventeen years and showed clinically as well as anatomically all the signs of this disease.

Several questions concerning lipoid nephrosis have been discussed. As at autopsy the kidneys were still much enlarged, it seems to be doubtful whether true lipoid nephrosis leads to contracted kidneys.

[21] Mason, E. H.: The Life History of a Case of Nephrosis, Internat. Clin. 1: 163, 1926.

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STUDIES ON THE BLOOD VESSELS IN THE MEMBRANES OF CHICK EMBRYOS

PART I. ABSENCE OF NERVES IN THE VASCULAR MEMBRANE

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PLATE 5

(Received for publication, April 23, 1930)

Experiments on the behavior of the blood vessels have with few exceptions been confined until now to tissue in which there are nerves. In all the organs of the body in adult human beings or in animals that have attained full growth, in which such investigations have taken place, the presence of vasomotor nerves has been demonstrated either anatomically or physiologically (1).

It is to be expected that in investigations of the reaction of blood vessels in tissues or an organ which is not innervated, the part usually played by the nervous factor can be ascertained. Experiments carried on in such preparations might make possible in a new way the elucidation of questions having clinical interest, as for instance, those arising in arterial hypertension (2) in which hypersensitivity of all vessels is present as well as in arteriosclerosis (3) in which the level of irritability is reduced and finally, in cases of inflammation (4, 5) in which the part played by the nervous system is still the subject of discussion.

On the Absence of Nerves in the Yolk-Sack of Chick Embryos

Concerning the occurrence of nerves in the membranes of chick embryos there exist in the literature, so far as we know, no references (6). There are, however, numerous investigations on the innervation of membranes of human beings and other mammals to which we intend to refer by way of comparison. The question whether the umbilical cord and the placenta contain nerves dates according to Schott (7) from the time of Galen. Reports of more recent investigations, to the literature of which Schmitt (8) has referred, and which are based in part on histological investigation carried out in tissues studied mainly by the method of impregnation with silver, and in part on pharmacological methods, contain the conclusion that the umbilical cord contains nerves of its own but only in that portion close to the body, and that its greater distal portion as well as the placenta are free. Ikeda (9) recently, at the suggestion of Aschoff, has reinvestigated this matter and has confirmed the statements of earlier authors.

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We have attempted to solve the question as to whether the membranes of chick embryos contain nerves in part by physiological, in part by histological investigations.

Physiological Experiments

Technique.—The experiments were conducted in a constant temperature chamber at 38°C. Sources of error due to fluctuation of temperature are therefore avoided. Eggs in all stages of development, but mostly those 3 to 4 days old, were studied. In older eggs the separation of the membranes is more difficult but can, after practice, be conveniently managed. Eggs were opened at the air-chamber end without turning or jarring them, as much of the shell as was necessary to expose the vascular region being removed by means of forceps. The embryo surrounded by sufficient of its membranes could by this means be exposed. The loss of albumen was, of course, prevented. The manipulation of the egg on opening it must be gentle; otherwise the blood vessels will be found even at the beginning of the experiment to be contracted, so that weak stimuli would lead, while they are in this state, to erroneous inferences.

For electrical stimulation the usual form of a reliable inductorium was used. The source of current was an Edison storage battery. When drugs were injected the point of the needle was inserted laterally until it came to lie under the membranes, since preliminary experiment showed that better and more uniform absorption took place than if these substances were applied drop-wise to the surface of the preparation. The experiments were brief, never lasting longer than 10 minutes so that errors due to desiccation might be avoided. The observations were made with a Zeiss dissecting microscope furnished both with binoculars and bin-objectives. The embryos were protected from excess heat by placing a water filter between the source of light and themselves.

We observed the behavior of blood vessels of the yolk membrane in some 1600 eggs at various ages. The experiments which we report now, selected from the great number which were performed, permit our making the following statements concerning the innervation of these vessels.

1. One locus of the membrane was touched several times with a fine

glass rod. Depending on the number of contacts and their strength, either constriction or dilatation took place. These stimuli brought about this effect both in capillaries and in small arteries. Even the strongest stimulus of this mechanical sort brought about no change in the immediate environment. The effect of the stimulus was strictly confined, wherever it was exercised, to the site which was stimulated.

2. A faradic current was employed to stimulate a given locus of the vascular layer, electrodes of varying diameters being used. Both the smallest arteries and the larger arterial branches were stimulated. One electrode consisted of cotton wool moistened with Ringer's solution which was placed on the membranes at a distance from the vascular zone, that is to say, beyond the marginal vein. The other electrode had the diameter of a hair. As the result of the stimulus, dilatation occurs either in a capillary or at one point of the artery, but is so small in extent as to be recognized only microscopically. If an electrode having a larger diameter is used the effect is apparent in a larger area. In all cases the effect of stimulation, whether it be contraction or dilatation, is to be recognized only at the point at which the stimulus is applied or, when the strongest current in our scale was used, in the immediate neighborhood. Involvement of neighboring regions or a propagation from the capillaries to larger arteries, or propagation in the reverse direction was never noticed.

The following experiment was made with the view to extending these observations and to detecting the possible existence of conduction over nerve paths:

The central end of an artery which led to a vascular area which we wished to stimulate was tied off and the whole vascular region was isolated by a circular incision so that every connection with the embryo was severed. In this isolated vascular region electrical stimuli were made just as before. The result of stimulation was the same as when the region was still in continuity with the embryo. On account of the absence of a circulation, that is to say of additional blood, the column of blood in the vessel when it was dilated, the degree of dilatation depending, of course, on the strength of the stimulus, appeared to be paler than before. Measurement of the diameter microscopically indicated, however, the same degree of dilatation as when the connection with the embryo was intact.

3. A fine hollow needle was inserted outside the vascular area and then pushed forward so that its opening came to lie beneath it.

Sodium iodide solution 6 per cent was injected in the amount of 0.1 cc. Dilatation of the vessels occurred at once but only at the point of injection. If larger amounts were injected a larger region was affected. If the same amount of saturated solution of sodium iodide is injected, stasis in the region of the drop immediately occurs. On account of the stagnation of blood which results, blood accumulates in the portion of the vessel central to this point. This appearance quite obviously represents a secondary phenomenon. The effect of the stimulus itself remains confined to the site of the stimulus. It appears to be unnecessary to report our experiments with other chemicals. The result was always the same—the stimulus affected merely the locus at which it was applied. If larger amounts were injected there was always the possibility that after absorption of the substance, and distribution in the circulation, a result would occur at points far removed from the point of stimulation and would there give the impression that a stimulus had been applied. But when small amounts are used and are confined to a small area the effect of the stimulus takes place only at the point of stimulation.

These observations differ from those of blood vessels which are known to be innervated. In the skin there occurs with every stimulus of low intensity an easily demonstrated erythema, an effect of the stimulus which can be seen far beyond the point of stimulation (Ebbecke (10), Lewis (11)). Reactions like this can be understood only as the result of an action in which the nervous pathways are involved. In the pancreas of rabbits for instance, Ricker and Regendanz (4) found that the effect of strong stimuli involved a larger region than the point stimulated in contrast to that of weaker oncs, the effect of which was merely local. In such cases the result is brought about by a reflex (nervous) action. In our experiments we could never bring about an effect beyond the region of stimulation even if strong currents were used. The restriction of the reaction of the blood vessels in the vascular areas in our preparation to the site of the stimulus suggests strongly that there are no nerves in the vascular layer of these embryos.

Among the drugs that have been tested which have an effect upon the blood vessels adrenalin is the only one which has an action conspicuously different from the ordinary on the vascular area of these membranes.

Adrenalin, in a solution of 1 per mille, was injected through a needle at a point just under the vascular area since it had been learned that adrenalin applied to the surface had no effect even when large amounts were used. 0.1 cc. of a 1 per mille solution brought about no visible contraction. It was not until an amount of 0.4 cc. or more had been injected that a contraction of the blood vessels took place, but even then the effect was never immediate. Indeed the earliest evidence of action was observed only after ½ minute. In most experiments contraction did not begin until the end of 4 to 5 minutes.

But even large doses like those just mentioned were not effectual in all cases. Of 38 observations, constriction took place in 25, in 4 the effect was uncertain, and in 9 there was no effect at all. Because of the weak action of the drug we were obliged to use relatively speaking unusually large amounts of fluid, 0.5 cc. or more. Control experiments with equally large doses with physiological salt solution of about the same hydrogen ion concentration, had as a matter of fact a very similar result. We are inclined to attribute the effect to the solvent therefore rather than to adrenalin. Even if we were to attribute the effect observed to adrenalin, the extent of it was remarkably small.

The contrast between this result and that in tissues which are normally innervated is striking. For in the latter it is easy to obtain a reaction everywhere by the local application of 0.1 cc. of a 1 per cent solution. The small effect in the vascular area of our preparations is accordingly altogether unusual.

Results similar to ours have been reported by Schmitt (8) in the case of blood vessels of the placenta which were treated by a perfusion method. If into the blood stream of the perfused placenta 1 cc. of suprarenin in the concentration of 1 to 1000 was injected, no effect was observed on the rate of flow in the vessels. Since it is generally recognized that the action of adrenalin takes place by virtue of its effect on nerves or on mechanisms of a nervous nature, the small effect of adrenalin on the vessels of the vascular membrane of chick embryos may be taken to represent further evidence that a nervous mechanism is wanting in these structures.

Anatomical Evidence

We have examined the embryonic membranes of chicks by means of a large variety of histological methods using the mesentery of rabbits as well as other organs, amongst them the skin, heart and lungs, by way of controls. Our best results were obtained by the use of Bielschowsky's method of silver impregnation in block, and also with the rongalite white method.

1. Investigations with Bielschowsky's Method.—We impregnated by Bielschowsky's method, 6 embryos and their membranes aged 3, 4 and 7 days, according to the recommendations of Schmorl (12), and cut them in serial sections. In the case of older embryos only single sections of the membranes were made. As early as 72 hours after the beginning of incubation, small, fine nerves were found in the region of the brain and in the ganglia of the head. At the end of 96 hours nerves could be found everywhere in the brain and spinal cord as well as in the spinal ganglia (Fig. 1). At the end of 7 days nerves were to be found in all parts of the embryo.

Contrary to the situation in the embryo itself, nerves were to be found nowhere in the membranes. In those sections in which the transition from embryo to membranes could be clearly seen it was apparent that the nerves were confined to the structure of the embryo and did not proceed over to the membranes, as for example in a specimen 7 days old (Fig. 2, b). Small single fibrils were seen stretching beyond the region of the lateral limiting sulcus (Fig 2, a) but in no section could they be traced to the membranes themselves. The last of the nerves found in Fig. 2 is shown by means of greater magnification in Fig. 3.

2. Investigations with the Rongalite White Method.—Whereas the silver impregnation method according to Schmorl's description gave always completely satisfactory results, difficulties were encountered at first with the rongalite white method. The only investigators who seem to have used this method have been Kreibich(13) and Glaser (14, 15). They succeeded even in cases where other methods failed in obtaining first-rate results both with intra- and supra-vital staining. Since an intra-vital technique was impossible on account of the small size of the objects, we have used the supra-vital method only. The mesenteries of rabbits were again used as controls. Since both the authors just mentioned have described the method in a cursory way only, we have decided to describe it in detail, according to the procedure which gave us the best results.

Rongalite white, which is methylene blue reduced by means of rongalite, was prepared in the following way: 1 gm. of pure methylene blue was dissolved in 200 gm. of distilled water, was heated, and then 6 gm. of rongalite and 15 drops of hydrochloric acid were added. This solution was boiled until it became clear and was then filtered. Staining was carried out in the following way. Organs were taken from the embryo immediately after death and were bathed in physiological salt solution. Rongalite white 5 per cent to 10 per cent was next added. After pieces of tissue had been stained for 45 minutes they were fixed in ammonium molybdate 5 per cent. After thorough washing they were placed in absolute alcohol and xylol and were then drawn up on to glass slides.

By this method we examined the membranes of 19 embryos of 3, 4, 8, 9, 10, 13, 18 and 20 days' incubation after they had been stained and a control provided for each membrane. Whereas in the mesentery, nerves could be beautifully demonstrated everywhere, none could be found in any of the membranes.

DISCUSSION

It appears then from our histological investigations that we were unable by means of the two best methods available to us to detect nerves in the membranes of the chick embryo. Neither did we find primitive cell processes which might be regarded as undifferentiated nerves. Our results therefore are in complete agreement with those which have been published in the case of the placenta both of man and of other mammals.

Is the conclusion to be drawn that the membranes contain no nerves? The objection may be made that since we are dealing with embryonal tissue we should not expect to find fully differentiated nerves. But the fact is that these tissues pass through a definite cycle of development, the course of which we have been able to follow from beginning to end, and that we have been unable to demonstrate nerves in any stage of their development, although the embryos themselves, from on the 3rd day on, always contained them.

Our anatomical results are in agreement with our physiological investigations. The absence of a reaction in the immediate neighborhood of an area, even when strong stimulation has been applied to the area itself, and the atypical behavior of the blood vessels in adrenalin experiments, can be understood only as resulting from the absence of nerves.

CONCLUSION

The agreement of physiological experiment with anatomical findings justifies our conclusion: the blood vessels of the vascular membrane of chick embryos do not contain nerves.

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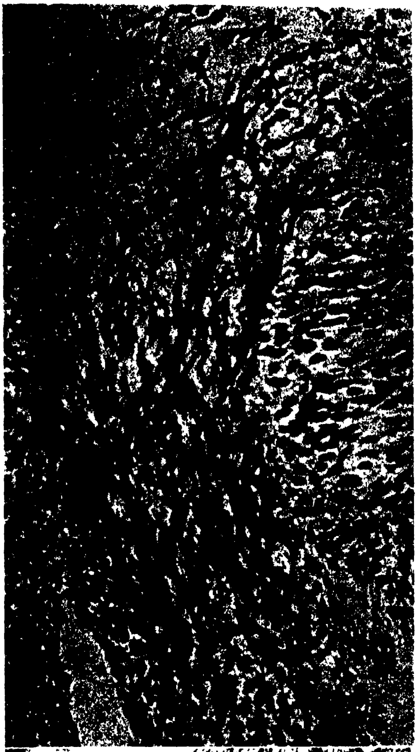
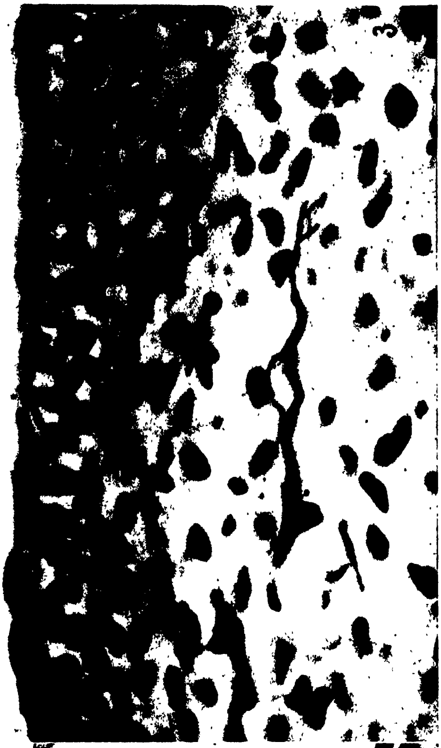
EXPLANATION OF PLATE 5

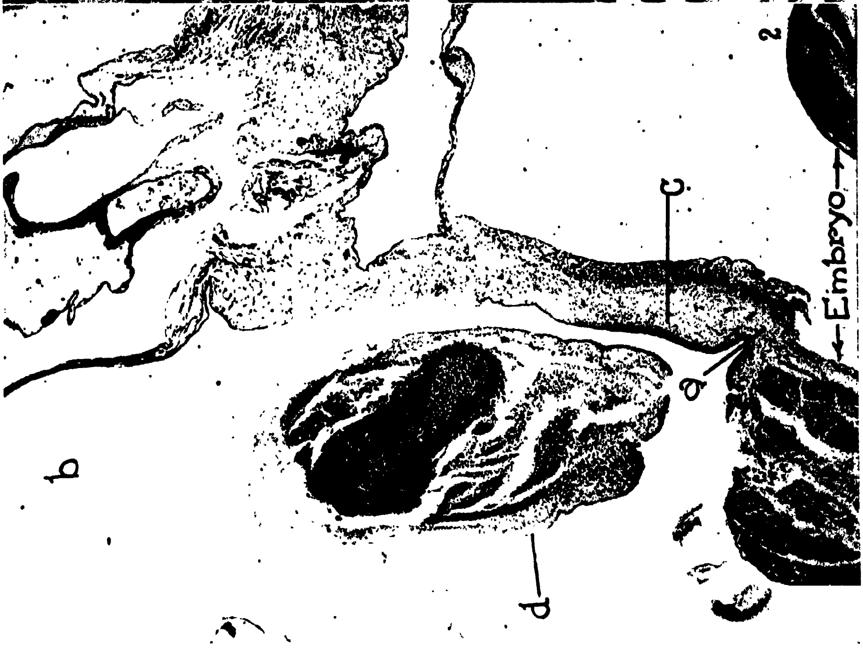
Fig. 1. Chick embryo No. 177. Incubation period 4 days. Neurofibrils in a ganglion-anlage of the body. Silver impregnation. × 450.

Fig. 2. Chick embryo No. 173. Incubation period 7 days. A cross section through the body is shown at the point of transition from the embryo to the membranes. At (a) the lateral limiting sulcus appears, and at (b) the amnion and chorion. The neurofibrils farthest away from the body (Fig. 3) were found at (c). At (d) there is a cross section of an extremity. Silver impregnation. × 40.

Fig. 3. The region (c) indicated in Fig. 2 is shown. × 1000. It exhibits the most distant peripheral nerve found in this section.







(Lange *et al.*: Blood vessels in chick embryos. I)

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STUDIES ON THE BLOOD VESSELS IN THE MEMBRANES OF CHICK EMBRYOS

PART II. REACTIONS OF THE BLOOD VESSELS IN THE VASCULAR MEMBRANES

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In recent years there has been lively discussion on the behavior of the vascular system especially concerning the dependence of reactions of the vessels on the nervous system (1, 2, 3, 4). In order to advance knowledge of this matter and to make clear what actually takes place, and to attempt to indicate the significance of these processes, it has become necessary to enlarge this study by examining a new and a greater variety of material. The blood vessels of the vascular membranes of chick embryos seem especially adapted to the ends in view. Membranes containing the vessels can be easily examined both microscopically and macroscopically after removing the shell and the shell membrane. Stimuli of any desirable kind may then be easily applied. Since it has been shown (Part I) that these tissues contain no nerves, the course of the reaction takes on a special interest.

A. The Effect of Electrical Stimuli of Varying Strength on the Blood Vessels

Faradic Stimulation.—The technique used was the same as that employed in Part I. The electrode consisted of two copper wires each a millimeter thick and separated a distance of 1 mm. by means of collodion. The points of the electrodes, which alone came in contact with the tissue, were polished together with the collodion in which they were embedded, by means of sandpaper. Eggs were used after having been incubated 4 days.

In order to facilitate comparison, stimuli were made in the following experiments always at the same place, namely, the final precapillary branches of the artery which passes upwards towards the head of the embryo and to the right.

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Experiment 1.—A very weak faradic current just strong enough to create a sensation of prickling on the moistened lips was used. After the electrodes had been placed, without exerting pressure, upon the appropriate vessels, current was allowed to flow through the electrodes for 10 seconds. They were then removed for 10 seconds in order to observe the effect which had been created, and then were replaced for a further 10 seconds. The duration of the stimulation was measured with a stop-watch. After the stimulus had been applied for 20 seconds, dilatation of the vessels appeared. The flow of blood in them continued to be rapid. In the surrounding vessels there was no change. We designate this stage in which there is dilatation with rapid flow "the stage of fluxion," adopting Ricker's term. 1 minute after the end of stimulation, the original diameter and rate of flow had returned.

From this experiment we made the following inference: The weakest possible faradic current brings about a brief stage of fluxion.

Experiment 2.—In this case a faradic current of medium strength which causes merely a burning sense when applied to the lips was used. The site of the stimulus was the same as in Experiment 1. The duration of the stimulus was 20 seconds, during the course of which there was an interruption of 10 seconds. At the end of 20 seconds, contraction of the blood vessels developed. The rate of flow was rapid. If the stimulus was continued for 20 seconds longer, the blood vessels shut. In the capillaries just peripheral, the flow ceased, so that the blood in them was motionless. Others were empty. The larger arteries central to these appeared unchanged. The flow of blood just proximal to the point of contraction was rhythmical. 5 minutes after the end of the stimulus the original diameter of the vessel was eestablished and the rate of flow was as before.

We made the following inference: A faradic current of medium strength brings about contraction of that arterial branch which has been stimulated.

Experiment 3.—A faradic current which was just strong enough still to be borne when applied to the lips, and which caused an unpleasant sensation of burning was used in the same way and at the same site as in Experiments 1 and 2. After 20 seconds there appeared a spindle-shaped dilatation, the degree of dilatation being greater than in Experiment 1. The rate of flow was at first slow, and then stopped after a number of seconds at a point further out than the site stimulated. Central to this point the dilated vessel was filled with blood. The blood corpuscles agglutinated so that they were no longer individually identifiable. Adopting Ricker's term we designate this "the stage of stasis." Capillaries peripheral to the site of stimulus were empty. Just central and just peripheral to that portion of the affected vessels which was dilated, but within the region of influence of the stimulus, the arterial branch was contracted; in consequence, the dilated portion appeared as distinctly spindle-shaped. The spindle-shaped dilatation and the contraction of that part of the vessel just central to it appeared to occur synchronously. The capillaries beyond, that is to say peripheral to the vessels so affected, were empty. Transition between the dark red region of stasis and that region beyond which appeared light owing to the absence of blood could be recognized macroscopically as a sharp line. Stasis persisted throughout the observation and could not be influenced by any new stimulus.

From these findings we have drawn the inference that a strong faradic current brings about stasis.

In order to ascertain whether and to what extent dilatation depends on the pressure of the inflowing blood stream, we carried out the following experiment.

We placed a pincette which shut off the flow of blood on a small artery. A stimulus of the same strength as that employed in Experiment 3 was then applied to the artery. Dilatation of the same degree took place. The blood in the dilated vessel was, however, a lighter red since it was distributed in a greater space. After 2 minutes the pincette was removed. The dilated vessel was thereupon filled with blood and the spindle appeared to be quite full.

There appears, therefore, to have taken place, genuine primary dilatation with filling as a secondary phenomenon. The dilatation may become so great and the spindle so filled with blood that in young specimens the total volume of blood contained in the embryos has been seen to collect in the dilated area. Under these circumstances the heart continued to beat but appeared as a mere shadow, seeing that it was empty of blood.

Under similar experimental conditions a weak faradic current brings about dilatation of the arterioles of the vascular membrane with increased rate of flow, the stage of fluxion, a current of medium strength, contraction to the point of closure, and a strong current stasis of the blood in a dilated vessel. It appears therefore that the reaction depends upon the strength of the current. It is necessary next to ascertain whether other stimuli have the same consequences.

B. The Effect of Various Other Stimuli

(*a*) *Mechanical Stimuli.*—In this case the stimulus consisted of touching the locus with a blunt glass rod. The site of the stimulus was as before. Because of the greater size of the glass rod, small arteries as well as the capillaries were involved.

The *gentlest possible* contact was established 3 times in 1 second, the intervals between being naturally brief. As a result the small arteries and capillaries dilated, becoming larger than those in the neighborhood. The rate of flow in the dilated vessels was rapid. This is the stage of fluxion. *Stimuli of medium strength* were applied 3 times, at the same rate as before and to the same site but with somewhat greater pressure. The small arteries and capillaries now contracted and disappeared. The site which was stimulated appeared as a lighter spot, empty of blood. The vessels in the surrounding region were not visibly altered and exhibited a rapid flow. 5 minutes later the small arteries again filled from the side of the heart and 1 minute later still the general appearance was the same as before the application of the stimulus. *Strong stimuli* consisted in bringing the glass rod in contact with the vessels 3 times, using quick strong strokes. An enormous dilatation of the small arteries and capillaries took place at once in the region stimulated. Centrally and peripherally, waist-like contractions were observed. The capillaries which were related anatomically to the area, but which were outside the region affected by the stimulus, were empty. An hour afterwards no change had taken place, stasis still persisting.

We drew the following inference: Depending upon the strength of the stimulus, mechanical irritation brings about a stage either of fluxion or one of contraction or one of stasis.

(*b*) *Ammonia.*—The site of stimulation was as before. The stimulating substance was applied through the needle of a small syringe.

Experiment 1.—When 0.5 cc. of a 1 per cent solution was injected, dilatation took place at once as well as a rapid flow of blood at the site of stimulation.

Experiment 2.—10 minutes after the stage of fluxion had been brought about as in the first experiment, the injection of ammonia (0.5 cc.) was repeated. The capillaries and the smallest arteries contracted. When a third injection of the same amount was made there was moderate stasis in the capillaries whereas the small arteries were contracted.

Experiment 3.—When 0.1 cc. of a 5 per cent solution of ammonia in Ringer's solution was injected slowly, the arterial branch which was affected dilated enormously at once while complete cessation of blood flow through it took place. Dilatation was so complete that stasis did not develop as in the previous experiment but instead coagulation of the blood. Agglutinated cells floated about in a clear medium within the vessels.

We have drawn the following inference: Ammonia brings about the stage of fluxion, of contraction, or of stasis and hemorrhage, according to the concentration used and the period during which it acts. Strong concentrations cause coagulation (pseudostasis).

We have chosen the experiments described as examples of the effects of 3 qualitatively different kinds of stimuli. But we have also made experiments with mustard oil, with silver nitrate, with potassium iodide and with oil of camphor. In principle the action of all was the same. It was possible by grading the concentration and the dose, to bring about the same effects as when the faradic current, the glass rod or ammonia was employed. Since the stimuli used were able to produce an effect on blood vessels in tissue in which there were no nervous elements, we have been forced to conclude that they have their effect upon the cells themselves and that these have an inherent power to exhibit the motions which we have observed.

The action of adrenalin as reported in the first of these papers was inconstant. Atropin had no action at all even in concentrations as high as 1 per cent. Cold and heat had no distinct effect. For that reason we were unable to utilize different degrees of temperature as means of establishing grades of reaction on the part of the vessels.

In our experiments we have found then that with effective stimuli there is a systematic relation between the strength of the stimulus and the reaction of the blood vessels, in the sense described by Ricker in the case of blood vessels in which a nervous apparatus exists. A weak current as we have shown is followed by the stage of fluxion, one of medium strength by that of contraction and a strong one by stasis. The general rule of the relatedness of a particular form of reaction to a particular intensity of stimulus is exhibited also by the non-innervated blood vessels studied in these experiments and by the kinds of stimuli which were employed. Our observations therefore confirm Ricker's theory concerning the laws governing the relation between reactions and the strength of stimuli. In the case of blood vessels which are not innervated it appears that the strength of the stimuli not the nature of the stimulus constitutes the determining factor. The same forms of reaction were found whenever it was possible to grade the strength of the stimuli irrespective of their kind.

C. The Effect of a Given Stimulus upon Different Portions of the Vascular System

In the experiments so far reported, stimuli were always permitted to exert their effect upon vessels of a like diameter. We have next to report our study of the effects of the same stimulus on capillaries, small arteries and on arterial main stems. For this purpose we have employed faradic current, mechanical stimulus, or one of the chemicals already mentioned, mustard oil, silver nitrate, potassium iodide or the oil of camphor.

Experiment 1.—We stimulated a capillary area with a very weak current for 20 seconds. There resulted the stage of fluxion. When the same stimulus was applied to small arteries or the main stems of arteries, no visible reaction took place and we drew the following inference: A stimulus which brings about the stage of fluxion in capillaries has no effect upon arteries.

In *Experiment 2* a stimulus of medium strength applied for 20 seconds to a small artery caused contraction. A stimulus of the same strength applied for the same length of time to the capillaries caused stasis. If the same stimulus was applied for the same length of time to an arterial stem, no distinct effect ensued although the appearance of a slight degree of dilatation occurred occasionally. From this experiment we draw the inference that a stimulus which causes contraction in small arteries causes stasis in the capillaries.

Experiment 3.—If a strong stimulus was applied for 20 seconds to an arterial stem, stasis took place in the vessel which had undergone spindle-shaped dilatation. The same stimulus if applied to small arteries or capillaries likewise caused stasis. And we draw the inference that a stimulus which causes stasis in arterial stems does so likewise in small arteries and capillaries.

SUMMARY

A comparison of the effect of stimulation upon the arterial subdivisions shows that these differ in irritability. The capillaries are most irritable, then come the small arterial branches, and last the main stems of arteries, these being the least irritable. The non-innervated blood vessels of the vascular membranes exhibit therefore the behavior described by G. Ricker in the case of those which have a nervous apparatus. We have demonstrated accordingly similar properties in vessels which are not innervated as in those which are. Certain differences exist however. The action of adrenalin on the vessels of the vascular membrane is inconstant and certain other substances are wholly ineffective. Ricker's experiments have advanced knowledge on the regularity of the vascular reactions and have paved the way for these experiments of ours. The result of our experiments with vessels free of nerves suggests that a reexamination may be fruitful of the mechanism which obtains in innervated vessels. There can be no doubt that nerves play a rôle in the behavior of vessels; it appears now to be necessary to define more accurately precisely what this is.

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STUDIES ON THE BLOOD VESSELS IN THE MEMBRANES OF CHICK EMBRYOS

PART III. ANATOMY AND PHYSIOLOGY OF THE BLOOD VESSELS AT DIFFERENT AGES*

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(Received for publication, April 23, 1930)

The development of the vascular system of the membranes of chick embryos passes through a definite cycle. It can be followed from its inception in the blood islands, through the stages in which the blood vessels are finally developed, to the time when it is discarded at the time of hatching. It ought to be possible therefore to correlate changes in function with the anatomical changes. We hoped to obtain an insight into the alterations which take place in both, and to do this the more readily owing to the short duration of the whole cycle.

Physiological Experiments

The irritability of the vessels was studied by applying stimuli of the same strength to them from their inception to the time of hatching. The experiments were carried out in eggs that had been opened as in Parts I and II of these studies.

It was necessary to introduce a slight modification in technique in the case of older embryos for in them it is more difficult to remove the membrane than in embryos 5 days old. The eggs were therefore kept on end, the air-chamber upwards, for 12 hours before the beginning of the experiment. With practise it became easy after opening the shell in the region of the air-chamber, to remove the shell membrane with fine forceps and scissors. The blood vessels then are as easy to approach as they are in younger embryos in which they are disposed along the long axis of the egg. The stimuli employed were a faradic current of known strength, sodium iodide, ammonia and mechanical stimulation with the use of a glass rod. As in our earlier experiments we ascertained which strength of stimulus it was necessary to employ in order to secure stasis in an egg after 3 days of incubation. We then chose a stimulus based on this, but weaker, maintaining all the stimuli which we employed at the same strength for all other ages. On account of the similarity of our observations it is necessary to describe only our results.

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At the time when there are only blood islands and no regular flow of blood, contraction and dilatation do not occur. At this early age the occurrence of coagulation as the result of the use of chemicals in strong concentrations can naturally not be observed. Our observations begin therefore with the behavior of blood vessels at the time when a regular rate of flow begins, namely, on the 3rd day. All stimuli which bring about stasis in arteries of the membrane on the 3rd day of incubation produce the same dilatation in the same length of time in arteries of the same size and in the same way in all eggs of all later ages. The criterion of a strong stimulus has been described in previous sections of these papers and may be defined as follows: In the case of a faradic current it is one which on moist lips causes an unpleasant sensation of burning. In the case of sodium iodide a concentration of 6 per cent in the amount of 0.1 cc. and in the case of ammonia a 5 per cent solution of the same amount. We chose a weaker current, as has been said, one which in small arteries causes contraction and studied the reaction to it through all ages up to the 19th day, that is to say, shortly before hatching of the egg. In all stages of development the effect of stimulation of arteries of the same caliber is the same, namely, contraction.

A difference in the manner of reaction is always visible. Capillaries react to weaker stimuli than do arteries. In this sense specimens which are younger differ from older ones. In specimens 3 and 4 days old, all the vessels respond to stimuli which in older embryos affect only those vessels which have the same small dimensions as the young ones. The larger vessels in older embryos require stronger or longer stimuli. For example, stasis was observed in all arteries in 3 to 4 day old embryos as the result of applying an electrical stimulus of a certain strength for 20 seconds. In specimens 18 days old on the contrary this stimulus applied to large arteries required twice the duration, namely, 40 seconds or a correspondingly stronger stimulus for 20 seconds to bring about the same effect. This striking difference is to be explained in terms of histology.

Anatomical Investigations

There are satisfactory investigations by Popoff (1) and H. Virchow (2) on the distribution and branching of blood vessels of the vascular membrane of chick embryos at various ages, but a comparative histological study of these vessels at all stages of development has not so far been published. In especial nothing is known on the presence of elastic tissue nor of the appearance of degeneration in the older aged groups. In the very early stages of development studies have been made by Sabin (3) both in living preparations and histôlogically. According to her investigations a continuous differentiation of mesenchymal cells to angioblasts takes place in the first 2 days. These cells develop first in the membranes which are of interest to us. Angioblasts differentiate further during the course of the 3rd and 4th days of incubation. The angioblasts give rise to endothelium, blood plasma and blood islands. Only those structures are called blood islands which develop hemoglobin and become erythroblasts. The lumina of the blood vessels come into being through a process of cytolysis either in the centers of the cell masses or in the cytoplasm of single cells.

Our histological description begins at the 3rd day of incubation when the flow of blood in the vascular membrane has become established.

Technique.—Freshly opened eggs were fixed in formalin, Mueller formol or Zenker's solution. Blocks of paraffin or of gelatin were then made which were cut in series from the marginal veins to the embryo. Specimens of various ages were so prepared, cut either horizontally or transversely. They were stained with hematoxylin and Van Gieson's solution, resorcin-fuchsin and Sudan hemalum.

Description of Embryos 4 Days Old.—Radiating from the embryo there are ridges and folds which press into the yolk. In these folds the blood vessels run. The arteries lie in the folds nearer the yolk. They consist of single rows of flat or spindle cells, the circumference of the wall being the length of 10 to 12 cells. In the surrounding area there is a loose mesh network in which there are large nuclei. These cells do not belong to the arteries since they are also to be found without relation to them. Within the vessels there are free nucleated cells either round or spindle-shaped depending upon the aspect which they present. Laterally from the arteries lie two veins. In comparison with the arteries they have a characteristic form. They are polygonal or flattened and adapt their shape to the space which is available, in this case, half surrounding an artery. Their wall is thin and consists of a single layer of cells. Arteries and veins are immediately juxtaposed so that at such places two layers of flat cells are to be found. There are no elastic fibers and no collagen. All vessels that belong to the yolk have the same form and are to be distinguished only by their size.

Description of Embryos 10 Days Old. Vessels at a Distance from the Embryo.—In the interval the dimensions of the arteries have increased to a five-fold extent. The wall turned towards the yolk consists of a single layer of cells. They contain a light staining protoplasm. The nucleus stains darker and contains many dark points arranged about a larger, more compact, area within. The relation of arteries and veins is less intimate; the veins run, at least in part, an isolated course.

Vessels near the Embryo.—Arteries have come to differ from veins through the distinct difference in thickness of their walls. On cross section the arteries appear to be round in contrast to the veins which are capable of taking on a variety of forms. The veins surround the arteries to the extent of a half to three-quarters of their circumference. The artery is embedded in the loose web-like tissue of the embryonic membranes. Their walls are about 5 cells thick. Their arrangement is lamellar. The nuclei are arranged in the long axis. The protoplasm appears more concentrated and is less in amount. The inner layer consists of somewhat taller endothelium. Between the cells delicate fibers stained bright red with Van Gieson's solution are to be found. There are no elastic fibers and no fat. The veins exhibit, surrounding the single row of cells of which they are formed, a layer of cells somewhat loosely meshed, the cells being large and composed of light staining protoplasm. Between the largest and the smallest arteries transitions occur ranging in dimension from arteries presenting two to three layers of cells to those of the largest size. The arrangement is always circular and never longitudinal. The smallest blood vessels, namely capillaries, often appear close to arteries, touching their outermost layers, but do not penetrate their walls.

Description of Embryos 14 Days Old. Vessels at a Distance from the Embryo.—The smallest arteries are built like capillaries and are to be distinguished from them only according to their size. They are of exactly the same form and structure as were those described in embryos of an earlier age. Nearer the embryo there are likewise to be found larger arteries of 2 to 5 layers.

Vessels near the Embryo.—A large artery of about 12 layers is to be found, the lumen being lined with a single layer of endothelium. Surrounding this is a longitudinal layer three cells thick. Beyond is a circular layer seven cells thick. Outside the vessel there lie connective tissue cells irregularly distributed and loose meshed, the cells containing small nuclei. The protoplasm of the cells in the arterial wall stains brown with hematoxylin and Van Gieson. These are young muscle cells. Distributed among them there are a few collagen fibers stained pale red. In the loose meshed tissue which is to be regarded as the adventitia there are a few collagen fibers but there are no elastic fibers and there is no fat. The veins have a larger lumen than the arteries and are built of single layers of endothelium and about two layers of cells. The cell layers appear to be looser meshed than in arteries.

Embryos 18 Days Old. Description of the Vessels Far Removed from the Embryo.—Arteries built like capillaries are to be found as well as small arteries such as have already been described.

Vessels near the Embryo.—The arterial trunk described in embryos 14 days old is to be found also here. The wall has become thicker. The nuclei are well stained and their structure unchanged. There is no evidence of nuclear degeneration, decrease in the size of the nuclei nor indeed of any other change. There is no fat in the wall nor any elastic fibers.

At the Site of Transition to the Embryo.—The form of the artery is unchanged. There appear to be more collagen fibers. Sections stained with resorcin-fuchsin exhibit stoutly developed elastic fibers. In the outer third of the muscle layer there lies a broad band of elastic fibers passing in part into the adventitia. This zone consists of about five layers of short twisted elastic fibers which form a meshwork. There is a second layer of elastic fibers very fine in structure, which forms the internal elastic layer close under the single layer of endothelial cells. This structure also is not closely organized, but consists of single short slightly curved fibers which run circularly. Neither fat nor any other form of degenerative substance is to be found. This part of the artery exhibiting elastic fibers should no doubt be reckoned as being part of the embryo. This is the portion in which, as has been described in Part I, nerve fibers were found.

In the embryo itself in the early stages there are definite elastic rings in arteries of very much smaller dimensions than in the vascular membrane. The proof that elastic fibers occur in arteries within the embryo, whereas in those without there are none, is certain since in one and the same section both the embryo itself and the vascular membrane are frequently found.

SUMMARY AND DISCUSSION

On the basis of the anatomical studies presented the following inferences or conclusions are drawn.

1. In the course of development there appear in the vascular membranes of chick embryos arterial vessels of all calibers, namely, capillaries, small arteries with 2 or 3 cell layers, and large arteries formed of endothelium, longitudinal and circular layers of muscle and adventitia.

2. In none of the stages are elastic fibers developed. Only in the most central portion of the umbilical artery, in that portion namely

which is to be regarded as belonging to the embryo, are elastic fibers discoverable.

3. The structure of capillaries is histologically the same at all stages. The small arteries of embryos 10 days old resemble histologically those of 18. At no stage of development are appearances of degeneration nor of fat to be found in arteries.

When the physiological results of our investigations are compared with the anatomical ones the following comments may be made. In respect to Paragraph 1 of the anatomical results we may remark that when we study the different forms of the wall of arterial vessels the most delicate vessels consisting of single cells exhibit the greatest irritability. Those which are built of 3 to 4 muscle layers are less irritable. Stouter vessels appearing for the first time at 10 days of incubation require stronger stimuli to bring about the same reaction. In respect to Paragraph 2 of the anatomical results we may make this comment. The absence of elastic fibers in all arteries of the embryonic membranes throughout the period of their development is important in defining a physiological property of the larger vessels.

The medium and larger vessels, beginning with the 4th day of incubation, contract differently from normal adult human arteries. In the contracted state they appear in cross section not as small replicas of larger circular structures, but take on a new form. During the course of contraction they become flat and appear band-like as would a garden hose when it is compressed by a weight. In examining a vessel so contracted one sees on rotating the vessel either a broad side or a narrow one. It is for this reason that such arteries appear alternately narrow as a line or broad as a band. It is not until the narrow artery is elevated with a hook that its uniform band-like nature becomes evident. The absence of elastic tissue, the presence of which in all probability is mainly responsible for the usual shape of arteries on cross section, permits one to see how the phenomenon which has been described may come about. Concerning Paragraph 3 of the anatomical conclusions we have this to say. According to the histological investigation a stage of degeneration is wanting in the blood vessels of the embryonic membrane in a sense in which one is accustomed to see such changes in other blood vessel systems during the course of life. On the day of hatching the constituent cells and fibers of the arteries of all calibers are anatomically the same as in their early development. These vessels do not die as the result of aging: The nutrient fluid ceases to flow because of contraction of the umbilical vessels. The blood vessels die in complete possession of their physiological irritability and anatomical integrity. The unaltered irritability of blood vessels of the same caliber at all ages is consonant with their unaltered anatomical structure.

We wish to express our thanks to Miss Nelda Ricci for technical assistance.

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STUDIES ON THE BLOOD VESSELS IN THE MEMBRANES OF CHICK EMBRYOS

PART IV. MODIFICATION OF IRRITABILITY OF THE BLOOD VESSELS

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We studied the reactions of the blood vessels in the vascular membranes of chick embryos in Part II of this series. There we described changes which took place quantitatively and qualitatively in response to stimuli. It was our care in the earlier experiments to maintain the environment constant. In the present experiments we intended conversely to keep the stimulus constant but to change the environmental conditions. The question we have asked ourselves is, of course, whether with the same stimulus a difference in the irritability of the blood vessels can be brought about by altering the environment.

To obviate the danger of drying, due to the increased length of time required by these experiments, a substitute procedure was devised. We placed the eggs in Ringer's solution through which oxygen was bubbled. It was, of course, necessary first to be certain that this arrangement did not interfere with the viability of the embryos.

Experiment 1.—Eggs were opened on the second day of incubation, the albumen for the most part was drained off, and the yolk was laid in the Ringer's solution through which, as has been said, oxygen was continuously bubbled. At the beginning of the experiment the embryo with its membranes was 11 mm. in diameter, the blood vessels were not yet developed, and the embryo itself white in color. 24 hours after the beginning of the experiment the embryo and its membranes measured 14 mm., the heart was red and contracted 120 times per minute. In the blood vessels there was flow of red blood. At the end of 48 hours the embryo and its membranes measured 16 mm., the heart beat at the rate of 120, and there was vigorous flow in the vessels. On the 3rd day, the rhythm of contraction of the heart was irregular.

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It is clear from these observations that the embryo including the circulatory apparatus grows normally for 2 days in this solution.

The demonstration of the viability of embryos and their membranes was sufficient to warrant the performance of experiments, the duration of which in no case exceeded 1 hour. We needed, of course, to be certain that oxygen in the concentration in which it was used exercised no effect on the irritability of the blood vessels of the embryonic membranes.

Experiment 2.—This was of a preliminary nature and was repeated in each case before proceeding to further tests. After opening the egg a branch of an artery was stimulated. Stasis occurred after the stimulus had been applied 3 times for 10 seconds. Then the preparation was irrigated with Ringer's solution through which oxygen was bubbled for 5 minutes. The vessel was again stimulated and stasis occurred again after 3 successive periods of stimulation each 10 seconds long.

We concluded that Ringer's solution through which oxygen was passed did not alter the irritability of the blood vessels.

We used carbon dioxide of known concentration in our experiments. It appears from the experiments of Hammett and Zoll (1) that saturated solutions of carbon dioxide bring about constriction of the vessels. Contraction takes place if the solution saturated with gas is injected under the membranes, as in the experiments of Hammett and Zoll, as well as when the surface is bathed with Ringer's solution which during this process is exposed to carbon dioxide in the usual fashion by bubbling the gas through the solution. These results we were able to confirm. We reduced, therefore, the concentration of carbon dioxide in Ringer's solution to a point at which it had no longer an effect upon the blood vessels.

Experiment 3.—Ringer's solution was placed in a glass burette having a capacity of 50 cc. Through this, air containing 5 per cent of carbon dioxide was bubbled so that 60 bubbles passed through in a minute. The egg after it had been opened was bathed with this solution. At the end of an hour the contractions of the heart remained unchanged, the rate being 128 per minute. No change took place in the diameter of the vessels, nor in the flow of blood through them.

We concluded that bathing the membranes with Ringer's solution containing carbon dioxide, the hydrogen ion concentration being between 7 and 6, would occasion no change in the rate of the heart or in the diameter of the vessels.

In view of the preliminary experiments which have just been described we adopted the following plan of operation.

A burette was provided with: (1) a tube through which oxygen was introduced, (2) a similar one for the delivery of carbon dioxide, and (3) a funnel through which salt solutions could be delivered. A tube led from the burette to a small glass cannula from which the solution designed to bathe the egg was permitted to drop. The perfusion stream was directed so that it fell not upon the embryo itself nor upon its membranes but on the surface at a distance from them, yet in such a way that the stream passed over the vascular layer. Direct impact of the fluid would have occasioned a mild stimulus the effect of which would have been characterized as we now know by dilatation of the vessels and a rapid rate of flow, the "state of fluxion."

Stimulation was supplied by means of a faradic current, the source being an Edison storage battery. To be certain that the current was constant, its voltage and amperage were continuously observed. The electrodes consisted of two copper wires a millimeter in diameter, placed a millimeter apart. With the exception of their poles they were carefully insulated. Observations were made with a binocular binobjective Zeiss microscope.

We measured the duration from the beginning of stimulation to the time of occurrence of stasis. To recognize this stage presented no difficulty because of the magnitude of the effect. Nor did this observation lead to error for the effect did not disappear and itself, therefore, served to control the observation. The experiments were all undertaken in a constant temperature room. Since the solutions and the gases were also kept here, the possibility of error due to alterations in temperature was excluded.

These arrangements permitted observations in which the vessels were stimulated alternately in milieux containing carbon dioxide and in others free of this gas.

After opening the egg: (1) Stimulation with a faradic current for 20 seconds was undertaken. The result was *stasis* of the blood stream. (2) When irrigation with Ringer's solution, *free* of carbon dioxide had been carried on for 2 minutes, stimulation with the faradic current was again applied for 20 seconds. *Stasis* again occurred. (3) When irrigation with Ringer's solution containing *carbon dioxide* had gone on for 3 minutes followed by stimulation with the faradic current lasting

150 seconds, *no stasis* appeared. (4) Next we irrigated the membrane with Ringer's solution which contained *oxygen* for 5 minutes and stimulated the vessels for 30 seconds. *Stasis* again appeared. (5) We irrigated again for 3 minutes with Ringer's solution containing carbon dioxide. This was followed by stimulation with a faradic current for 200 seconds, but *no stasis* occurred. Finally, we irrigated the preparation with Ringer's solution containing oxygen for 5 minutes and now stimulation with faradic current for 30 seconds only, was followed by the occurrence of *stasis*. Forty experiments like this were performed.

DISCUSSION AND SUMMARY

When faradic stimulation was undertaken of vessels irrigated with Ringer's solution, which alternately contained and was free from carbon dioxide, it was observed that the reaction was far less when the solution contained carbon dioxide. A reversal of the effect could be obtained many times. It appears, therefore, that when Ringer's solution contained carbon dioxide in the concentration described, the irritability of the vessels to electrical stimuli decreased, although carbon dioxide by itself and in the absence of the application of the stimuli, appeared to be void of effect upon the vessels. The rare, divergent results were traced to technical errors.

We attempted to discover whether the observed decrease in irritability of the vessels might not be due to the absence of oxygen. For this purpose we irrigated the vessels with Ringer's solution alternately containing nitrogen and oxygen. When nitrogen caused any change this was due to an influence on the rate of the heart and not on the irritability or reactivity of the arteries. In whatever way we tried we were unable to bring about a change in reactivity of the arteries by creating a condition of oxygen lack independently of a change in the rate of the heart beat.

We attempted to study also the effect of other acids beside carbon dioxide on the changed reactivity of the arteries. Irrigation with various concentrations of lactic acid was without result. We also employed solutions buffered with potassium and sodium phosphate. When the irrigation was undertaken with these solutions having a pH range varying from 7.7 to 5.9 we observed neither a direct action nor one which modified the preparation in such a way as to change its susceptibility to faradic stimulation.

Important investigations have been published recently by Atzler and Lehmann (2) on the direct influence of the hydrogen ion concentration on the behavior of blood vessels. Hammett and Zoll believed that, as the result of their experiments in which they attempted to bring about stimulation with solutions of concentrated carbon dioxide, they were able to exclude the possibility of action due to acid alone and therefore ascribed to carbon dioxide a specific effect. In our own experiments the method of irrigation does not permit an inference whether, or how far, an acid effect plays a rôle in the carbon dioxide experiments. For beside the question of hydrogen ion concentration and of buffering, the question of the penetration of substances from the surface to the contractile elements of the wall of the vessels requires to be considered. Carbon dioxide has an ability, beyond that of all other substances, to penetrate through tissues (3). It may be owing to this property that we could influence the reactivity of the blood vessels with it and it alone.

This possibility must be further investigated. In these experiments, however, it was our object to show only that it was possible to influence the irritability of blood vessels experimentally. The conclusion is justified by our experiments that carbon dioxide in small concentrations reduces the threshold of irritability for electrical stimuli of the blood vessels of the embryonic membrane.

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HEMOGLOBIN, THE HEME PIGMENTS, AND CELLULAR RESPIRATION

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Hemoglobin has long been studied as a carrier of oxygen in the blood of vertebrates. More recently (1885) a pigment related to hemoglobin was observed by MacMunn in the cells of a great variety of lower animals which have no hemoglobin either in their cells or body fluids. After long neglect, MacMunn's observations were recalled, and were confirmed and extended. In addition still another pigment was discovered which is related to hemoglobin and which in cells acts as a respiratory catalyst. A group of pigments called heme pigments and closely related to hemoglobin is accordingly now known. They exist in all aerobic cells where one of them, at least, plays an essential rôle in cellular oxidations. This review is concerned with the chemical properties and biological behavior of heme pigments. Hemoglobin will be included in the discussion because knowledge of the pigments is largely based on a wide study of hemoglobin and its derivatives. Hemoglobin itself is a compound of a colorless protein, globin, and an iron porphyrin pigment. In general the pigments formed by the combination of iron with the various porphyrins are called hemes.

The functions of the heme pigments have attracted investigators with very varied interests so that they have been studied from many different points of view. In organic chemistry their constitution has been of interest; in physiology their chemical properties were analyzed first in order to understand the respiratory functions of the blood, and more recently in connection with the problems of cellular respiration; in physics, at least in the case of one investigator, they have been studied because of their clearly defined spectroscopic properties; in pharmacology because of their reactions with drugs and poisons; and in natural history because of their wide distribution in nature.

These investigations of the heme substances may be considered to have been along four lines.

1. The investigations of the organic chemists (Hoppe-Seyler, Nencki, Piloty, Küster, Willstätter and Hans Fischer) on hemoglobin present a continuous development from the time of Hoppe-Seyler to the present. Although until recently the knowledge gained has been of relatively little physiological significance, the physiologist now has problems that will cause him to seek the aid of organic chemistry. How, for instance, do the heme-like pigments differ from the heme of hemoglobin, and how are the porphyrins of the hemes related to the porphyrins of chlorophyll? The similarity in constitution between heme and chlorophyll becomes more interesting to the physiologist now that he is aware that heme is a universal cellular constituent. Investigation of the porphyrins is at present very active, due mainly to Hans Fischer and his school. Only the bare outlines of these extensive and thorough investigations can be given in this review. Fischer has written several general accounts (1924, 1929a).

2. The physiologist has studied the intact heme molecule from the point of view of hemoglobin, for this was until recently the only heme substance of any known physiological significance. When the other heme pigments occurring in nature were discovered, the question of how globin modifies the properties of heme in hemoglobin was expanded into the general problem of how heme is influenced by combination with nitrogen compounds. Much of the information acquired in the study of hemoglobin (for instance the knowledge of the reactions between carbon monoxide and hemoglobin) was simply applied to other heme compounds.

3. Naturalists, or chromatologists, as they often called themselves (such men as Sorby, MacMunn and Lankester), interested in the colors of the whole animate creation, added the spectroscope to the equipment of the naturalist, and examined plant and animal pigments wherever they could find them. MacMunn discovered heme-like substances in the cells of animals from sponges to vertebrates. These investigators had, what is so often lacking in physiologists, an interest in all forms of life. On the other hand, they failed to carry out any effective experiments. Perhaps it was for these reasons that they failed to influence the physiological study of hemoglobin. Indeed,

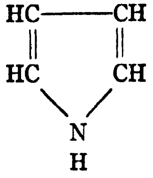
these researches of the chromatologists, which were confined to the nineteenth century, were not appreciated, and were nearly forgotten. Finally, however, it was the observations of MacMunn that led to the contemporary study of the cellular heme pigments.

4. A fourth way of investigating heme pigments has recently been developed by Warburg. The activity of these substances as respiratory catalysts is inhibited when they combine with carbon monoxide, but is restored when the carbon monoxide compound is dissociated by light. The behavior of heme catalysts can therefore be studied by measuring the oxygen uptake of systems exposed to known pressures of carbon monoxide and to light of definite wave lengths. By this method, heme pigments can be investigated in the living cell as well as in vitro.

Following the four points of view just outlined, this review will deal first with the chemistry of porphyrins, secondly with the chemistry of hemochromogen, thirdly with the distribution in nature of the heme pigments, and fourthly with the heme pigments as respiratory catalysts.

I. CHEMISTRY OF THE PORPHYRINS. The starting point for investigations of the porphyrins has usually been *hemin*, familiar as Teichmann's crystals. This is the hydrochloride of heme, the protein-free part of hemoglobin. Heme has a molecular weight of about 650 (Fischer and Hahn, 1913) which was measured directly by the effect of heme on the boiling point of pyridine. This estimate is the same as the minimum molecular weight calculated on the basis of the iron content of heme.

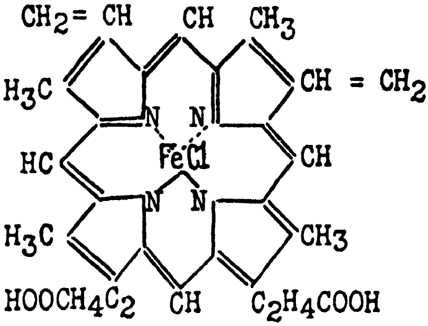
The problem of the constitution of heme was first studied by splitting it into its component parts and studying the parts. For this purpose heme has been broken down by oxidation, by reduction with acetic acid and hydrogen iodide, and by alkyl splitting. The products which were obtained are all derivatives of pyrrol



and have all been synthesized so that their structures are known. Some are pyrrol acids, others are pyrrol bases. A detailed study of these decomposition products showed that there are four pyrrol nuclei in heme, of which two are bases and two acids. Possible structures of heme were then proposed by Küster, Willstätter and Fischer. At the same time Fischer began his investigations on the synthesis of hemin.

1. *The Synthesis of Hemin.* Fischer's investigations were based on knowledge not only of the building blocks of heme, pyrrol derivatives obtained by a relatively vigorous decomposition, but also on an acquaintance with substances obtained from hemin by gentle treatment. The essential step in the preparation of these substances, the *porphyrins*, is the splitting off of iron from the heme molecule. At the same time the side chains of the pyrrol nuclei are modified so that a number of different porphyrins can be prepared differing from each other in the constitution and arrangement of these side chains. Hematoporphyrin was the first porphyrin to be obtained in crystalline form (Nencki, 1900) and many have subsequently been crystallized, each having its characteristic crystal form, melting point and absorption spectrum. Some of the porphyrins obtained from heme occur in nature. The porphyrin whole structure most closely related to that of heme is protoporphyrin with which iron can recombine to form a hemin indistinguishable from that prepared directly from hemoglobin. The porphyrins seem, indeed, able to combine with iron to form a series of hemes, which can be crystallized as hemins. The iron-porphyrin compound of aetioporphyrin (Willstätter, 1912), for instance, would be called aetioheme, its hydrochloride, aetiohemin. Aetioporphyrin is of special interest since it can be prepared from both hemoglobin and chlorophyll.

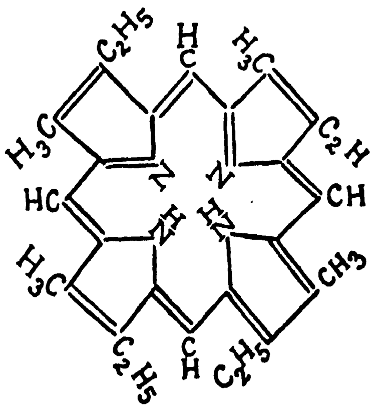
Because of the close relationship between the porphyrins and hemin, and because of the pyrrol building blocks of hemin were already identified, Fischer's first step in the synthesis of hemin was naturally an attempt to join several pyrrol nuclei together to form a porphyrin. The first pyrrol compounds made did not have the properties of a porphyrin, even when they contained as many as four pyrrol nuclei; later, however, methods were discovered of combining four pyrrol nuclei in such a manner as to form a porphyrin (Fischer and Scheyer, 1924). Many porphyrins were synthesized, some of which had already been prepared from hemin but some of which were new. To make protoporphyrin it was necessary to introduce two unsaturated side chains. The addition of iron completed the synthesis (Fischer and Zeile, 1929). Fischer's formula for hemin is:



There are several points of biological interest to be mentioned in connection with the synthesis of hemin: The difficulty in introducing the unsaturated side chains was that the porphyrin was relatively inert chemically. To render it more active, the porphyrin was converted by adding iron into the corresponding heme to which side chains could then be joined. Iron was then withdrawn, alterations were made in the side chains to make them correspond to those of protoporphyrin, and finally iron was restored. Apparently an iron porphyrin is more active than a simple porphyrin. The free porphyrins which are found in nature are perhaps mainly excretory products.

In the synthesis of hemin, isomerism also presents a matter of biological interest. The hemin of hemoglobin is one of a number of isomers. Of the porphyrins now known to occur in nature some (viz. oöporphyrin, the porphyrin of egg shells) have the same configuration as the porphyrin of hemoglobin (Fischer and Kögl, 1923) and others (viz., turacin, a copper porphyrin present in the feathers of certain birds) a different configuration (Fischer and Hilger, 1923). The natural porphyrins now known are all constructed after the manner of two of the four possible arrangements. How the porphyrins of the cellular heme pigments are to be classified in this respect is not yet known.

2. *Heme Pigments and Chlorophyll.* In the study of the porphyrins the goal of the organic chemist has been not only the synthesis of hemin but also an understanding of the relationship between hemoglobin and chlorophyll. Before anything was known about the structure of either hemin or chlorophyll, suggestions were made that they had much in common. The first experimental evidence for the supposition was the preparation of a porphyrin from chlorophyll (Hoppe-Seyler, 1879, 1880, 1881). The nature of the relationship became clearer when Willstätter and Stoll (1913) prepared aetioporphyrin from both chlorophyll and hemoglobin.



Though this porphyrin is closely related to hemin, lacking only two unsaturated side chains and an iron atom, its relation to chlorophyll is obscure. Willstätter and Stoll considered it unlikely that chlorophyll itself contains the porphyrin nucleus, but rather that porphyrin is formed during decomposition. They were able to separate the decomposition of chlorophyll into a number of definite stages, each of which was characterised by the development of a crystalline substance. Of these the *chlorines* form an important group, marking a relatively early stage in the breakdown of chlorophyll. When the chlorines themselves break down, porphyrins are formed. The recent studies of Hans Fischer and his pupils throw a new light on the relationship between these two groups of substances, for they have been able to prepare chlorines also from porphyrins (Fischer, 1929b). Reducing a porphyrin with an alcoholate converts it into a chlorine, though by this simple procedure only a small yield is obtained. Since combination with iron renders a porphyrin much more active, the reaction was tried on various hemes (i.e., iron porphyrins), iron being subsequently removed. Chlorines were in this way more easily prepared. Because of the near relationship between chlorines and porphyrins, Fischer thinks there is no longer any doubt that chlorophyll contains the porphyrin nucleus itself or a system closely related to it. It would be a remarkable fact if two substances of such importance in cellular metabolism both contained the porphyrin nucleus, the one, chlorophyll, associated with the formation of sugar, the other, heme, associated with the combustion of sugar.

II. CHEMISTRY OF HEMOCHROMOGENS. The porphyrin substances active in cellular respiration are not simply porphyrins and probably not merely hemes (i.e., iron-porphyrins); the respiratory catalysts are probably hemochromogens, and the cellular heme pigments actually visible are certainly hemochromogens. Since hemochromogens seem to play an important part in cellular metabolism their chemistry is of considerable biological interest and will be dealt with in detail.

1. *Spectroscopic Properties.* Investigation of the hemochromogens has been largely dependent on their spectroscopic properties. In fact much of the knowledge available of all those substances mentioned in this review—porphyrins, chlorines, hemes, etc.—was obtained with the spectroscope because these pigments have unusually sharp absorption bands and because exact spectroscopic measurements are easily made. It is not only the exact position of each band but also the arrangement of the bands, their pattern, that is characteristic of each group of these substances. Each group can be identified by its own absorption pattern. It was for this reason that Hans Fischer supposed he had prepared chlorines from porphyrins, the absorption bands of the new substances being arranged in the same pattern as those of the "natural" chlorines prepared from chlorophyll by Willstätter. Nearly every step in the synthesis of porphyrins was guided by the spectroscope, though in every case information of other kinds was subsequently gathered. That spectroscopic knowledge should be reliable and its significance profound is amply explained by modern physics. It is naturally desirable to make spectroscopic measurements as precise as possible in order to distinguish between closely related compounds.

There are, for instance, isomeric porphyrins whose bands lie approximately in the same position, indeed to within 10Å[1], so near together that it seemed necessary to resort to melting point determinations to distinguish them (Fischer and Schormüller, 1929). Though 10Å is the limit of accuracy attainable for these bands with an ordinary spectroscope, they could perhaps be measured with an error not greater than 1 or 2 Å with the reversion spectroscope (Hartridge, 1912). The absolute amount of light absorbed at any particular wave-length should, moreover, also be measured, for only by doing so can a substance be completely characterized spectroscopically.

The importance of spectroscopic investigation of the heme pigments can only be fully perceived when one attempts to study these substances within the living cell. In chemical physiology it is frequently a most perplexing problem to find a way of observing and measuring the chemical changes which occur in living cells without changing the cell at the same time. The situation closely resembles that in astronomy where the chemistry of a body can be studied only from a distance. Chemistry of the stars has been made possible by the use of the spectroscope; in the same way, the chemistry of cells, in so far as it is concerned with substances having clearly defined spectroscopic properties, can be investigated. In the spectroscopic study of cells, as of stars, interpretation of observations depends upon an acquaintance with the spectroscopic properties of the substances involved. Consequently in discussing the chemistry of hemochromogen, emphasis must be laid on spectroscopic properties.

Kirchoff's discovery in 1859–60 of the explanation of Frauenhofer's lines in the solar spectrum, pointed the way to a knowledge of the chemical composition of the sun and stars; in the same year Bunsen applied the principles of spectrum analysis to the examination of terrestrial matter. Two years later Hoppe-Seyler observed the absorption spectrum of hemoglobin (Hoppe-Seyler, 1862). Hoppe-Seyler's paper was called to the attention of Stokes, the eminent physicist, who later wrote, "I had no sooner looked at the spectrum, than the extreme sharpness and beauty of the absorption bands of blood excited a lively interest in my mind, and I proceeded to try the effect of various reagents." The result was a study that has been the basis for all subsequent spectroscopic investigation of the heme pigments (Stokes, 1863–64). The experiments on hemoglobin, for instance, demonstrated that the difference in color between arterial and venous blood is essentially due to the difference in color between oxygenated and reduced hemoglobin.

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[1] Angstrom units.

When Stokes reduced[2] an alkaline solution of what we now call heme, he discovered a substance having two intense, sharp, absorption bands, and when he reduced a solution of hemoglobin previously warmed in alkali, the same spectrum was obtained. The substance with this absorption spectrum was called hemochromogen by Hoppe-Seyler[3] (1870).

2. *Chemical Nature of Hemochromogens* (Anson and Mirsky, 1925a, 1928). The hemochromogens are a series of compounds with the same absorption spectrum pattern but with absorption bands in different positions. A hemochromogen is formed when reduced heme combines with one of a number of nitrogen substances. Reduced heme, prepared in the absence of a nitrogen compound, is unmistakably different from a hemochromogen: in the region in which hemochromogen has intense, sharp absorption bands, reduced heme shows weak, diffuse absorption; under conditions under which hemochromogen is soluble, reduced heme is insoluble; and, of perhaps greatest interest biologically, combination with a nitrogen substance makes heme a much more effective oxidative catalyst. The differences between hemochromogens formed from the same reduced heme are due to the different nitrogen compounds with which the reduced heme is combined. Many, though by no means all, nitrogen compounds can combine with reduced heme to form hemochromogens. Cyanide, ammonia, amines, proteins, pyridine, nicotine, etc., have this property. The compounds appear to have only nitrogen in common. A hemochromogen is named by using the name of its nitrogen substance as a

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[2] Stokes used an ammoniacal reducing agent.

[3] Hoppe-Seyler substituted this name for that used by Stokes because he believed that as the person who had first isolated the substance in crystalline form he had the right to name it, though later he admitted having been mistaken in claiming to have crystallized hemochromogen. (Hoppe-Seyler and Thierfelder, 1893.)

prefix, as for example, pyridine-hemochromogen, ammonia-hemochromogen. The hemochromogen most frequently observed is that prepared directly from a hemoglobin solution by adding alkali and a reducing agent (sodium hydrosulfite, for instance). The nitrogen substance which this hemochromogen contains is denatured globin, and it is therefore called globin-hemochromogen. If globin is separated from heme before adding the reducing agent and heme then reduced with sodium hydrosulfite, reduced heme, not hemochromogen is obtained. If globin is now added it combines with reduced heme so that the absorption spectrum of heme disappears and that of globin-hemochromogen takes its place.

The ability to form hemochromogen seems to be a general property of hemes (iron-porphyrins). Mesoheme and aetioheme, for instance (formed by the union of iron with mesoporphyrin and aetioporphyrin respectively) combine with nitrogen substances to form mesohemochromogens and aetiohemochromogens (Willstätter, 1912).

3. *History of Hemochromogen.* The account of hemochromogen just given differs so much from the views of the chemistry of hemochromogen until recently generally accepted, that some historical statement is needed. Since the discovery of hemochromogen by Stokes in 1864 the substance has always been identified by its absorption spectrum. That hemochromogen was simply reduced heme was accepted as a matter of fact by Stokes and subsequent investigators, though we now see that it was a theory based on the fact that when Stokes reduced heme he obtained hemochromogen. Stokes was unaware of the fact that when heme was reduced it also combined with ammonia in the reducing system used (for Stokes used ammoniacal reducing agents), to form a hemochromogen. Indeed it was soon found by Hoppe-Seyler that some reducing agents did not form hemochromogen from heme unless ammonia, albumin, or some other "organic" substance was added, which substance, it was thought, enhanced the reducing action of the system (Hoppe-Seyler, 1878). Consequently some reducing agents, particularly hydrazine hydrate (a nitrogenous one, we may note) were held with favor as being the proper reducers for heme. When hemochromogen was not formed it was thought that no reduction had occurred; reduced heme had not yet been discovered. In 1893 Bertin-Sans and Moitessier discovered reduced heme and realized that this turns into one or other of the hemochromogens "by the action" of ammonia, amines, and proteins (Bertin-Sans and Moitessier 1893a, 1893b). Whether "by the action" was meant an effect on the reducing agent or on the reduced heme was not stated. Subsequent investigators (Laidlaw, 1904) thought of the experiments of Bertin-Sans and Moitessier as having confirmed Hoppe-Seyler's view that a weak reducing agent is rendered effective by the presence of

protein. As for the existence of reduced heme as distinct from hemochromogen, this was confirmed by some investigators (Menzies, 1894) and not by others (Laidlaw, 1904, Hasselbalch, 1909). (Dilling appears to have agreed with Haselbalch.) Dilling (1910) went so far as to show that the hemochromogens crystallized from pyridine, piperidine and a number of similar bases differ from each other. His interpretation of the differences observed was that hemochromogen is an acid that can combine with various bases to form a series of esters.[4] Dilling thought that only secondary differences between the hemochromogens, such as the small differences in the positions of absorption bands, are due to combination of these bases "with the *pre-existing* hemochromogen radicle."[5] This view that hemochromogen is essentially reduced heme continued to be universally held as is illustrated by the fact that in 1923 Hans Fischer and Schneller (1923a), being able to add iron to porphyrins, tried to make hemochromogen simply by adding iron in the absence of oxygen. Finally it was definitely shown by Anson and Mirsky (1925a, 1928) that hemochromogen is not simply free reduced heme but is a compound of heme and a nitrogen group and that the particular hemochromogen prepared from hemoglobin is a compound of globin and heme. These conclusions are now generally accepted. (Hill and Holden, 1926; Roche, 1929.)

4. *Hemochromogens as Definite Compounds.* The clearly defined spectroscopic properties of the hemochromogens indicate that these substances are not vague complexes. Additional evidence that they are definite compounds is the fact that from solutions of pyridine-hemochromogen and some similar hemochromogens, crystals can be prepared (Donogany, 1893; Dilling, 1910) which by examination with a microspectroscope are proven to be hemochromogen crystals (Anson and Mirsky, 1928). Not all hemochromogens, however, have been crystallized.

5. *Shifts of Absorption Bands.* Under given conditions, the positions of the absorption bands of a hemochromogen prepared from a definite nitrogen compound are constant and exactly reproducible. In some cases, however, large shifts in position can be produced which are associated with precipitation of the hemochromogen (Anson and Mirsky, 1925a). These changes in position are probably caused by aggregation of the hemochromogen molecules (Keilin, 1926). In

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[4] Dilling's theory of ester formation is disproved by the facts that ester formation is impossible in the strongly alkaline solutions in which hemochromogens exist, that cyanide, which forms a typical hemochromogen is not a base and that hemes not possessing free carboxyl groups can form hemochromogens.

[5] The italics are ours.

general, aggregation of a pigment causes its absorption bands to shift towards the red end of the spectrum (Stenger, 1888, Svedberg, 1909). The correlation between dispersion and spectrum is easily observed with globin-hemochromogen (Mirsky and Anson, 1929a). If this hemochromogen is precipitated by salting out in alkaline solution or by neutralization, the band shifts some twenty-five Å toward the red end of the spectrum. The shift is always in this direction and may in some hemochromogens be as much as 170 Å. (Keilin, 1926.)

6. *Hemochromogen Equilibria* (Anson and Mirsky, 1925a, 1928). In a hemochromogen the nitrogen compound is loosely combined with reduced heme so that some uncombined nitrogen compound is always present, there being an equilibrium between reduced heme, free nitrogen compound and hemochromogen.

$$\text{Reduced heme} + \text{Nitrogen compound} \rightleftharpoons \text{Hemochromogen}$$

This equilibrium is closely analogous to the equilibrium formed when hemoglobin combines with oxygen for there, too, some free oxygen is always present.

$$\text{Hemoglobin} + \text{Oxygen} \rightleftharpoons \text{Oxyhemoglobin}$$

In the hemoglobin system the existence of an equilibrium is demonstrated by the fact that although only one molecule of oxygen is combined with one atom of iron in oxyhemoglobin, to convert all of the reduced hemoglobin into oxyhemoglobin, more than the equivalent amount of oxygen must be added. An excess of oxygen must be present to drive the above equilibrium to the right. Similarly, an excess of nitrogen compound must be added to reduced heme to convert all of it into hemochromogen, to drive the hemochromogen equilibrium to the right.

The analogy may be followed even further. Hemoglobin combines with carbon monoxide as it does with oxygen, a difference being that much less free carbon monoxide than oxygen is needed to obtain combined hemoglobin. The affinity of hemoglobin for carbon monoxide is, as is well known, greater than for oxygen. Similarly, the affinity of reduced heme for cyanide, for instance, is greater than for pyridine or ammonia. When hemoglobin is exposed to oxygen and carbon monoxide at the same time, the two gases compete for the hemoglobin.

$$HbO_2 + CO \rightleftharpoons HbCO + O_2$$

In the same way, different nitrogen compounds compete for reduced heme.

$$N_1 \text{ hemochromogen} + N_2 \rightleftharpoons N_2 \text{ hemochromogen} + N_1$$

Hemoglobin and hemochromogen equilibria are both affected by temperature and acidity. Hemochromogen when heated is dissociated into reduced heme and nitrogen substance. The affinity of reduced heme for a nitrogen substance increases as the solution becomes more alkaline. The precise effect of hydrogen ion concentration depends, to some extent, on the nature of the nitrogen substance, some requiring a definitely alkaline solution to combine with reduced heme, others being able to combine in approximately neutral solutions.

In studying hemoglobin and hemochromogen equilibria, the same methods can sometimes be used. Thus, ammonia-hemochromogen dissociates in a vacuum into reduced heme and free ammonia as does oxyhemoglobin into hemoglobin and oxygen. Although the methods originally used for the study of hemoglobin equilibria were gasometric and colorimetric, more recently spectroscopic methods have been used too. The equilibrium in which oxygen and carbon monoxide compete for hemoglobin can be studied quantitatively with a spectroscope simply by measuring the changing positions of an absorption band (Hartridge, 1912). The analogous hemochromogen equilibrium in which two nitrogen substances compete for reduced heme can be observed in precisely the same manner.

7. *Stoicheiometry of Hemochromogen.* The equilibria just described complicate the problem of finding the number of molecules of a nitrogen compound combining with one molecule of reduced heme to form a hemochromogen molecule. Analyses of "isolated" hemochromogens were made before hemochromogen equilibria were discovered, but we have no assurance that the substance analyzed was pure hemochromogen (von Zeynek, 1898, 1910; Pregl, 1905). Since a hemochromogen exists only in the presence of free nitrogen substance, it is hardly possible to isolate the hemochromogen uncontaminated by free nitrogen compound or by free reduced heme. It is also possible that a nitrogenous base in addition to acting as a nitrogen compound to form a

hemochromogen, might as a base combine with the acid groups of heme. Analysis of "isolated" hemochromogen would include both forms of nitrogenous base.

If in the equilibrium

$$\text{Reduced heme} + \text{Nitrogen compound} \rightleftharpoons \text{Hemochromogen}$$

the affinity for nitrogen compound is so great that the amount of *free* nitrogen compound is negligible compared with the amount of *combined* nitrogen compound, then the *total* nitrogen compound (which can be known) will be practically the same as that combined. Hill (1926, 1929) found that the addition of two molecules of pyridine is required to convert one molecule of reduced heme into hemochromogen. He believes that in his experiments the free pyridine was negligible, that all the pyridine added was combined with heme, and that therefore hemochromogen contains two nitrogen groups per heme. It is our opinion (Mirsky and Anson, 1929a; Anson and Mirsky, 1930b) that it was not proven that in Hill's experiments the free pyridine was negligible, for the aggregation of both reduced heme and pyridine hemochromogen complicates to an unknown extent the study of the pyridine hemochromogen equilibrium. If all the pyridine added was not combined with heme, then pyridine hemochromogen contains less than two pyridine groups per heme. From the study of the reactions of heme with cyanide the simplest conclusion is that hemochromogen contains one nitrogen group per heme.

8. *Reactions of Heme with Cyanide* (Anson and Mirsky, 1928, 1930b). It has long been known that cyanide reacts with reduced heme to form a pigment with a two-banded spectrum similar to, but definitely different from that of ordinary hemochromogen (Oppenheimer's Handbuch, 1909). A closer study of this reaction has shown that just one molecule of cyanide reacts with one molecule of reduced heme to form a compound which has a typical hemochromogen spectrum, and then still more cyanide reacts with this hitherto unobserved first cyanide compound of reduced heme to form the familiar second cyanide compound. Hill (1929) who has confirmed these results, has also shown that the second cyanide compound contains two cyanide groups per heme. The experiments with cyanide are simpler than those with pyridine because cyanide has a much greater affinity

for reduced heme than pyridine so that it is relatively easy to make the free cyanide negligible.

Experiments on the reactions of cyanide with globin hemochromogen show that cyanide reacts with globin hemochromogen to form a compound of globin hemochromogen and cyanide. In this reaction cyanide does not compete with or displace globin and so is not reacting like a typical nitrogenous substance. Cyanide can then further react with cyanide-globin hemochromogen to form the same second cyanide compound of reduced heme which is formed in the absence of globin.

We have, then, these facts, that the first cyanide compound of reduced heme contains one cyanide group per heme and has a typical hemochromogen spectrum, that the second cyanide compound does not have a typical hemochromogen spectrum, and that cyanide can combine with hemochromogen without reacting as a typical nitrogenous substance. So it seems simplest, for the present, at least, to conclude that in the absence of other nitrogenous substances, cyanide first combines with heme to form a typical hemochromogen containing one nitrogen group per heme and then combines with this cyanide hemochromogen just as it does with globin hemochromogen, this second reaction having nothing to do with hemochromogen formation.

9. *Combination of Heme with Proteins.* Proteins, as nitrogenous substances, combine with reduced heme to form hemochromogens. Denatured globin combines with reduced heme to form globin-hemochromogen, native globin combines with heme to form hemoglobin.

a. *Proteins as complicated nitrogen substances containing a large number of free nitrogen groups.* One cannot know in advance which and how many of these groups combine with reduced heme. A single molecule of denatured globin (hypothetical molecular weight 16,700) can convert at least 10 molecules of heme into hemochromogen, an estimate made by adding more and more reduced heme to denatured globin until there appears in addition to the hemochromogen bands, the characteristic absorption bands of reduced heme (Anson, 1926; Mirsky, 1926; Anson and Mirsky, 1928; Holden and Freeman, 1929). It takes much more edestin than globin to convert a given amount of heme into hemochromogen and more zein than edestin. The low hemochromogen-forming capacity of zein may be connected with its low diamino acid content. (Anson and Mirsky, 1928.) Similar differences between other proteins have been observed (Holden and Freeman, 1929).

b. *The difference between globin-hemochromogen and hemoglobin.*[6] One difference between these two compounds of globin and heme is that hemochromogen is a compound of heme and denatured globin, while hemoglobin is a compound of native globin (Anson and Mirsky, 1925d; confirmed by Hill and Holden, 1926; Haurowitz, 1929; Roche, 1929, and others). There may be still other differences between hemoglobin and globin-hemochromogen. It is not yet known whether these two substances have the same molecular weight or in precisely what way heme and globin are combined in either hemochromogen or hemoglobin. It is known, however, that in neither compound is the linkage through the unsaturated side chains of heme (Hill and Holden, 1926).

c. *The significance for protein chemistry of combination with heme.* Since hemoglobin is a compound of heme with native globin, and hemochromogen one with denatured globin, we have here a specific chemical criterion of whether globin combined with heme is native or denatured, an indicator as it were, of the state of the globin. The globin prepared by the classical method of Schulz (1898) is denatured globin not, as was originally supposed, the native globin of hemoglobin (Anson and Mirsky, 1925d, confirmed by Hill and Holden, 1926). After this was made clear, methods were devised for preparing native globin (Hill and Holden, 1926; Anson and Mirsky, 1930a). Though the remarkable physico-chemical differences between native and coagulated (or denatured) proteins are at once obvious, little is known of the underlying structural differences. Recent experiments indicate that the coagulation of hemoglobin, whether by heat, acid, or surface action, is reversible, for from completely coagulated hemoglobin a crystalline protein can be prepared having the same physico-chemical properties (solubility, coagulability, etc.) as the original native hemoglobin. There must also be "reversal" of some specific chemical groups, for the "reversed" protein combines with heme to form a hemoglobin whereas the coagulated protein combines with heme to form a hemochromogen. These recent investigations (Anson and Mirsky, 1925d, 1929b, 1930a; Mirsky and Anson, 1929b, 1930), on the reversal of protein coagulation can only be referred to here, but they suggest ways in which the types of linkage between heme and globin can be used in studying some important protein reactions. Holden and Freeman (1929) claim that coagulable proteins can form hemochromogens only when they are denatured.

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[6] Based on the old theory that hemochromogen and reduced heme are identical was the further theory which, so far as we know, had never been questioned until 1925, that when hemoglobin is converted into hemochromogen, globin is split off from the iron pyrrol complex, heme.

d. *The "synthesis" of hemoglobin.* Hemoglobin has been formed from heme and globin. Bertin-Sans and Moitessier (1892, 1893b) who first did this were careful to ascertain by sensitive spectroscopic tests that the globin they used was free of heme and the heme free of globin. From such globin and heme, methemoglobin, reduced hemoglobin and oxyhemoglobin were prepared. Bertin-Sans and Moitessier tried to prepare hemoglobin crystals "which would give a definite sanction to their point of view," that they had in fact prepared hemoglobin, but in this respect they were unsuccessful. The synthesis has been repeated several times (Menzies, 1915; Anson and Mirsky, 1925a; Hill and Holden, 1926) and since then synthetic hemoglobin and methemoglobin have been crystallized (Anson and Mirsky, unpublished experiments).

10. *Hemochromogen Reactions with Carbon Monoxide and Oxygen.* a. *Carbon monoxide.* Hemochromogens form loose combinations with carbon monoxide in much the same way that hemoglobin does.

$$\text{Hemochromogen} + \text{CO} \rightleftharpoons \text{CO Hemochromogen}$$

In the case of globin-hemochromogen this equilibrium (in the dark) is exactly described by the simple mass law equation. (Anson and Mirsky, 1925a.)

$$\frac{[\text{Hemochromogen}]\,[\text{CO}]}{[\text{CO Hemochromogen}]} = \text{K}$$

b. *Effect of light on carbon monoxide-hemochromogen.* The remarkable discovery of Haldane and Smith (1896) that light tends to dissociate carbon monoxide-hemoglobin has been found applicable also in the case of carbon monoxide-hemochromogen (Anson and Mirsky, 1925a Krebs, 1928). The combination of reduced heme itself with carbon monoxide is unstable in the light, but the instability is greatly increased when a nitrogen substance is joined to reduced heme, that is to say, carbon monoxide hemochromogen is more sensitive to light than is reduced carbon monoxide heme (Krebs, 1928).

The reactions with carbon monoxide are of fundamental importance for investigating the rôle of heme as a respiratory catalyst in the cell, for the CO compounds of heme are in general catalytically inactive and the CO compounds of the cellular heme pigments are extraordinarily sensitive to light. This sensitivity is closely approached by the substance formed when methyl carbylamine[7] combines with carbon monoxide-hemoglobin (Warburg, Negelein and Christian, 1929). Combining with this simple cyanide derivative renders carbon monoxide-hemoglobin several thousand times more sensitive to light. It is not known in this case whether carbylamine acts as a nitrogen substance, in the same manner as when combining with reduced heme to form a hemochromogen.

c. *Oxidation of hemochromogen.* Though hemochromogen, like hemoglobin, combines loosely with carbon monoxide, unlike hemoglobin, it is not known to combine loosely with oxygen. It reacts with oxygen and other oxidizing agents to form a substance analogous to methemoglobin, the oxide of hemoglobin. Hemoglobin and methemoglobin form a reversible oxidation-reduction system (Conant, 1923). When reduced hemoglobin, hemochromogen and reduced heme are formed from their oxides, one equivalent of hydrogen per atom of iron is required (Hill and Holden, 1927). The influence of its nitrogen substance on the tendency of a hemochromogen to oxidize is not known.[8]

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[7] Methyl carbylamine ($CH_3N = C$) was used instead of cyanide ($HN = C$) because the latter is an acid.

[8] Recently Conant and Tongberg (1930) have studied the oxidation-reduction potential of the reduced heme-oxidized heme system and have discovered that combination with nitrogen groups influences the potential. The calculations of oxidation-reduction potentials from the electromotive force measurements is made uncertain by the fact that the oxidation-reduction equilibrium is influenced to an unknown extent by the aggregation of the pigments (as Conant and Tongberg realized) and also by the fact that the relative extents to which pyridine combines with reduced and oxidized heme under the conditions of the experiments are not known. The complication of the pyridine-hemochromogen equilibrium by the aggregation of the pigments has already been considered in connection with spectroscopic experiments (Mirsky and Anson, 1929a; Anson and Mirsky, 1930b).

In general the conditions affecting the autoxidation and oxidation of the heme compounds await investigation. In the past observations on the so-called autoxidation of hemochromogens have been made only in strongly alkaline solutions containing oxidized products of reducing agents.

10. *Parahematin and hematin.* (Keilin, 1926.) The oxide of hemochromogen, the compound formed when a nitrogen compound combines with oxidized heme, is known as parahematin. It has a characteristic absorption spectrum of two rather diffuse bands. Perhaps the difficulty in recognizing the less sharply defined spectrum of parahematin accounts for the fact that knowledge of hemochromogen came earlier than that of parahematin. The concept of loose combination of reduced heme with nitrogen compounds to form hemochromogen can be extended with little change to oxidized heme with which the same nitrogen compounds form a series of parahematins. Changes in acidity may affect differently the combinations of reduced and oxidized hemes with nitrogen compounds. Globin-parahematin dissociates completely when a faintly alkaline solution is made tenth normal, in which solution globin-hemochromogen would scarcely dissociate at all (Keilin, 1926). On the other hand, according to Holden and Freeman (1929) denatured egg albumin forms a parahematin (as well as a hemochromogen) in normal sodium hydroxide, a parahematin which dissociates as the hydroxyl ion concentration is decreased.

The globin compound of oxidized heme has been known for some time (Arnold, 1900; van Klaveren, 1901) as cathemoglobin:[9] globin-parahematin would now be a better name, the name indicating that it is one member of a series of compounds. The name hematin has been used for about a century (Berzelius, 1840) often in a vague way, for a number of hemoglobin derivatives in which globin was supposed to be separated from heme, and the same name was used later for a solution of hemin. The substances obtained when an excess of acid or alkali is added to hemoglobin are known as "acid hematin" and "alkaline hematin" respectively and a neutralized form as "neutral hematin." We have seen that "neutral hematin" is not free hematin and is now known as parahematin. There has never been any proof that the heme in acid hematin and alkaline hematin prepared by adding acid and alkali to hemoglobin is in the same state as in a solution of pure crystalline hemin, and it is to avoid the confusion of calling different things by the same name that the pure iron-porphyrin is designated as heme. There can be little doubt, moreover, that the heme in solutions of acid or alkaline hematin is not free, for free heme is insoluble in acid and the spectrum of alkaline hematin differs from that of free heme in alkali. The heme of acid and alkaline hematin is neither free nor in clearly defined compounds such as hemochromogen and parahematin but probably occurs in the form of colloidal complexes with globin (for alkaline hematin, see Wu, 1926; for acid hematin see Keilin, 1926).

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[9] It is curious to note that Dilling thought that when cathemoglobin is reduced to hemochromogen, globin is separated from reduced heme—so firmly established was the idea that hemochromogen is reduced heme.

III. THE HEME PIGMENTS IN NATURE. 1. *The Relations Between the Heme Pigments.* The study of the spectra and other properties of known heme compounds *in vitro* with which this review has so far been concerned provides the essential basis for the knowledge that heme compounds exist universally in nature, in all aerobic cells, and that one of them at least plays an essential rôle in the catalysis of biological oxidations. Hemoglobin itself is not a catalyst of oxidations but a passive carrier of oxygen and it is not universally distributed. It is present in all vertebrates. Outside of the vertebrates, however, hemoglobin is found only here and there, sometimes in the blood and sometimes in the tissues, of totally unrelated groups of animals. For instance, hemoglobin exists in the larvae of a few insects but in no other insect larvae and in no adult insect and it is found in a star fish but in no other similar animals. Apparently a very peculiar iron compound has arisen independently in nature many times.

In addition to hemoglobin there occur occasionally in nature a few other pigments which are related to hemoglobin in that they either are hemochromogens or can easily be converted into hemochromogens. Helicorubin is found in snails (Dhéré and Vegezzi, 1917a, 1917b, 1919; Dhéré and Baumeler, 1926), actineohematin in the actiniae or sea anemones (MacMunn, 1885b) and chlorocruorin in certain worms (Milne Edwards, 1838; Lankester, 1867, 1870; Krukenberg, 1880 and 1882; MacMunn, 1885a, 1889a; Griffiths, 1892a, 1892b). The hemochromogens obtained from these pigments are not identical spectroscopically with the hemochromogen prepared from hemoglobin. *A priori* such differences may be due either to differences in the iron-porphyrin nuclei or to differences in the nitrogenous substances to which the iron-porphyrin nuclei are attached. It is possible to decide between these two possibilities by displacing the unknown nitrogenous substances with a known nitrogenous substance such as pyridine and comparing the spectrum of the resulting hemochromogen with that of pyridine hemochromogen made from the heme of hemoglobin. When this is done actineohematin and helicorubin yield the same pyridine hemochromogen as does the heme of hemoglobin (Anson and Mirsky, 1925b), while chlorocruorin yields a pyridine hemochromogen with bands distinctly further to the red (Fox, 1926a, 1926b). The heme of chlorocruorin can be converted not only into a hemochromogen but also into the other typical heme derivatives. There are then to be found in nature a number of true heme pigments, like hemoglobin of limited and haphazard distribution. Some of them, like actineohematin and helicorubin, yield the same heme as hemoglobin. Others, like chlorocruorin, yield a different heme.

2. *The Universality of Heme.* Of much wider distribution than any of the pigments so far mentioned is the histohematin of MacMunn (1886, 1887), which is related to and convertible into hemochromogen. MacMunn observed the sharp four-banded spectrum of histohematin in a great variety of animal tissues. For instance, in many echinoderms, molluscs, insects and worms as well as in the higher animals. Most of the animals in which histohematin was shown to exist do not contain hemoglobin. The important and easily verifiable observations of MacMunn were neglected for a generation because of the opposition of a great authority, Hoppe-Seyler (Levy, 1889; Hoppe-Seyler, 1890a, 1890b; MacMunn, 1889b, 1889c, 1890). Recently the study of histohematin has been revived by Keilin (1925, 1927) who has shown that the distribution of the pigment is even wider than MacMunn supposed (see also Bierich, Rosenbohm and Kalle, 1927; Yaoi and Tamiya, 1928; Yaoi, Tamiya and Nakahara, 1928). It is present in plants and in unicellular organisms such as yeasts or bacteria as well as in animal tissues. Keilin accordingly substituted for the name histohematin the more general name cytochrome.

The question naturally arose whether the more widely distributed heme-like pigments have the same iron-porphyrin nucleus as the occasional hemoglobin or the occasional chlorocruorin. Baker's yeast and the wing muscles of bees (which do not contain hemoglobin) were accordingly extracted with pyridine, the purpose being as with helicorubin and chlorocruorin to displace the unknown nitrogenous substances with pyridine. From both yeast and muscle two different pyridine hemochromogens were obtained, one indistinguishable from the pyridine hemochromogen derived from hemoglobin, the other similar to the hemochromogen derived from chlorocruorin (Anson and Mirsky, 1925b). It would be desirable to repeat these experiments with the new method now available (Anson and Mirsky, 1930a) and first to separate beyond doubt the hemes from the unknown nitrogen compounds. More recently crystalline hemin identical or at least isomeric with the hemin from hemoglobin has actually been obtained from yeast (Fisher and Schwertel, 1928).

Hemoglobin, it now appears, may no longer be regarded as the preeminent heme pigment, nor is its haphazard distribution now so mysterious. Hemoglobin is merely an occasional specialized derivative of an iron substance, heme, which is much more widely distributed. Similarly the nucleus of the rare chlorocruorin, or a heme very like it, is present in aerobic cells generally. It would be interesting to know whether hemocyanin, the copper pigment which carries oxygen in the bloods of some crustacea, is also an occasional derivative of a universal copper compound. There is evidence (Phillipi, 1919) which still requires confirmation, that the pigmented part of hemocyanin is actually a copper porphyrin. Unfortunately hemocyanin and its derivatives do not have the intense and well defined spectra of the heme pigments. It is probably for this reason that so little is known about them.

3. *The Hemoglobin Model.* If hemoglobin has lost some of its relative importance amongst the heme pigments, it has certainly gained in interest as a *type* of biological molecule worthy of the most detailed study. Heme itself cannot combine with oxygen loosely and so cannot act as an oxygen carrier. The biological fitness of hemoglobin is the result of radical modifications in the properties of heme resulting from its combination with globin (Anson and Mirsky, 1925a). It now looks as if all the heme pigments (perhaps enzymes, too) are built on

the hemoglobin model. The heme pigments in nature all have in common similar iron-porphyrin nuclei. They all, however, have individual peculiarities due in part to the specific natures of the substances to which they are joined. Certainly this arrangement is an economical means of utilizing the varied potentialities of a single kind of substance.

4. *Cytochrome.* a. *Distribution.* That cytochrome exists in almost all aerobic cells has already been mentioned. In general the four spectroscopic bands of the pigment are more intense the more intense the metabolism of the tissue (MacMunn, 1886; and especially Keilin, 1925). The spectrum of cytochrome is, for instance, very intense and easy to observe in the extremely active wing muscles of bees which can contract several hundred times a second. Exact measurements of the concentration of cytochrome have, however, not been made. It is not even known whether the apparent intensities of the bands are a reliable measure of the concentration of the pigment.

b. *Oxidation and reduction.* Cytochrome can be oxidized and reduced by suitable chemical agents. The reduced form has the four bands while the spectrum of the oxidized form is practically invisible. Oxidation and reduction can also take place in the living cell even without the addition of oxidizing and reducing agents (MacMunn, 1886). Yeast and a transparent moth show the phenomena most readily (Keilin, 1925). If the yeast is well aerated its cytochrome is oxidized. If the yeast is exposed to an oxygen-free atmosphere its cytochrome is reduced. In the case of the moth when the insect is active the cytochrome is partially oxidized. When the insect is quiet, its cytochrome becomes reduced again. Narcotics which influence the oxidation and reduction systems of cells naturally influence the oxidation and reduction of cytochrome (Keilin, 1925). In the presence of indifferent narcotics such as the urethanes, for example, cytochrome remains oxidized. Apparently the urethanes inhibit the reducing system of the cell. In the presence of small concentrations of the specific inhibitors of cellular oxidations, cytochrome remains reduced even if the tissue is well aerated. Cytochrome is apparently not autoxidizable. Its oxidation is catalyzed by the cyanide sensitive oxidation ferments. The effect of cyanide may be very nicely observed spectroscopically in the transparent moth. When the insect is inactivated by cyanide vapors the cytochrome in the wing muscles becomes reduced. As the insect recovers after removal of the cyanide vapors, the cytochrome too recovers and may be oxidized once more.

c. *Composition.* MacMunn (1886) realized that cytochrome is related to hemochromogen. He knew that when cytochrome is oxidized its sharp bands practically disappear, just as is the case with hemochromogen. And by the action of alkali he was able to obtain from cytochrome a typical hemochromogen. Nevertheless, MacMunn was not able to explain the exact relation of cytochrome to hemochromogen, nor to account for the fact that cytochrome has four bands while hemochromogen has two.

Keilin (1925) has analyzed the matter further. He has shown that cytochrome is not a single four-banded pigment but a mixture of three different hemochromogens. Three of the four bands are the three α bands of the three hemochromogens. The fourth band, the one farthest towards the blue, is the result of the fusion of the β bands of the three hemochromogens. Keilin's reasons for believing that cytochrome is a mixture, not a single pigment are these: 1. The relative intensities of the bands vary from the cytochrome of one tissue to that of another. This could not be true of a single pigment. It could easily be true of a mixture, the relative concentrations of the components of which are variable. 2. When cytochrome is oxidized and reduced the four bands do not disappear and reappear simultaneously. 3. Water extracts from yeast a single hemochromogen with an α band corresponding to one of the bands of cytochrome. 4. Cytochrome in general cannot be extracted as such. The complex nature of cytochrome has also been shown by a study of its reactions with pyridine and ammonia (Anson and Mirsky, 1925b).

None of the components of cytochrome react with CO in neutral solution, although they do combine with CO in strongly alkaline solution. Two, surely, of the components of cytochrome are not autoxidizable since in the presence of cyanide they are not oxidized by oxygen (Keilin, 1925).

The precise nature of the iron-porphyrin nuclei of the three components of cytochrome has not yet been decided although, as has already been stated, there is some evidence that there are two different hemes in cytochrome, one similar to the heme of hemoglobin, the other similar to the heme of chlorocruorin. Several porphyrins (Fisher and Fink, 1924; Fisher and Schneller, 1923; Schumm, 1925, 1926a, 1926b, 1926c) have been obtained from yeast. If these could be shown to have their origin in definite components of cytochrome then, of course, the nature of these porphyrins would provide valuable evidence concerning the nature of the hemes of cytochrome.

Keilin (1926) has succeeded in producing from ordinary pyridine hemochromogen a pigment or mixture of pigments with a spectrum similar to that of cytochrome. He believes his procedure to consist partly in the modification of the porphyrin by oxidation and reduction and partly in the precipitation of the pigment.

5. *Free Hemochromogen and Heme.* Some tissues which do not contain cytochrome in visible amount show a simple two-banded hemochromogen (MacMunn, 1886; Keilin, 1925). Many tissues, including those which do contain cytochrome, contain a free heme which is not visible but which can be made visible by suitable means (Keilin, 1926).

6. *Heme and Tissue Iron.* It has long been known that iron exists in tissues generally not as free iron but in some unidentified complex form. Since there are hemes present generally, some, at least, of this iron must be joined to porphyrin. There is evidence, not by any means conclusive, that about a third of the iron of baker's yeast and the wing muscles of bees is in the form of the same heme as that in hemoglobin (Anson and Mirsky, 1929a). The estimates of heme as an extremely small fraction of the total tissue iron (Von Euler, Fink and Hellstrom, 1927; Treibs, 1927) are definitely due to a technical error (Anson and Mirsky, 1929a).

IV. THE HEME PIGMENTS AND RESPIRATION. 1. *The Inhibitor Technique.* a. *The old heavy metal theory.* Substances which are stable when exposed to oxygen outside of the organism, within the organism are burned by oxygen readily. There must therefore be catalysts in the organism which speed up oxidations. Perhaps the greatest success so far attained in the study of biological oxidations has been the discovery of the nature of one of these catalysts. It has long been believed that an essential respiratory catalyst is a heavy metal compound, probably an iron compound, since iron is the heavy metal present in tissues in the highest concentration (Warburg, 1928). This conclusion rested on the fact that on the one hand respiration can be inhibited reversibly by minute concentrations of HCN or H_2S (Negelein, 1924), while on the other hand, heavy metal compounds can catalyze *in vitro* the oxidations of biological substances, but become catalytically inactive when combined with CN and H_2S. The inhibitor technique with HCN and H_2S had two great virtues. It permitted the study of the catalysis of respiration without any irreversible injury, serious disturbance, or permanent change in the tissue which was being studied. It resulted in the discovery that a catalyst essential to normal respiration has some of the characteristic properties of the heavy metal compounds. In fixing, however, the precise nature of the heavy metal compound acting as a respiratory enzyme, the old inhibitor technique and, in general, the experiments based on the old heavy metal theory were a failure. Warburg's original evidence that the heavy metal involved is iron was, in our opinion, completely unconvincing. And even if the respiratory ferment were an iron compound, the old experiments could not decide what sort of an iron compound—an important matter since the properties of an iron compound depend to a large extent on the nature of the complex of which the iron is a part. As a result of the apparently inherent limitations of the inhibitor technique in its old form, progress in the search for the nature of the heavy metal respiratory catalyst simply stopped.

b. *The new methods.* The new investigations of Warburg (Warburg and Negelein, 1929d), however, have revolutionized the study of biological oxidations. The old inhibitor technique with its great and unique virtues of not involving any permanent injury to the cell and permitting the study of normal respiration has been retained. But to it there has been added the new virtue of specificity. And as a result it has been demonstrated that an essential respiratory enzyme is not only a heavy metal compound but in particular a heme compound. This great and sudden change has been brought about because, following the newer study of the heme pigments in nature and *in vitro*, the inhibition of respiration has been taken up from the point of view of the particular properties of the heme pigments rather than from the point of view of the general properties of heavy metal compounds and because Warburg has added to the old purely qualitative inhibitor technique an entirely new, quantitative and powerful kind of spectrum analysis.

c. *Inhibition by CO.* The new qualitative facts are these. The respiration of tissues may be inhibited by CO in the dark completely and reversibly. By the action of visible light this inhibition may be abolished. Iron-porphyrin compounds *in vitro* like the respiratory ferment *in vivo* can combine with CO reversibly to form CO derivatives which may be dissociated by visible light. These iron-porphyrin compounds can catalyze oxidations. Their CO derivatives are catalytically inactive. Outside of the iron-porphyrins no other substances have as yet been shown to react reversibly with CO to form light-sensitive derivatives. It still remains possible, however, that some other kind of iron compound as yet unknown or unstudied reacts with CO in just the same manner as the iron-porphyrin compounds or the respiratory ferment, and that the respiratory ferment is this other kind of compound. This uncertainty cannot be avoided in the qualitative form of the inhibitor technique.

d. *The new spectrum analysis.* So far the experiments with CO have been exactly the same as those with CN, the only advantage of CO being that it is a more specific although still not an absolutely specific inhibitor. The part of Warburg's present procedure which yields an entirely new *kind* of information is the spectrum analysis which permits the demonstration not only that the respiratory ferment has some peculiar properties also possessed by iron-porphyrin compounds but that the respiratory ferment definitely *is* an iron-porphyrin compound. Light of one color is more effective in influencing the CO inhibition of respiration than light of another color. This means that the CO compound of the respiratory ferment is a pigment which absorbs one kind of light more than another. Suitable quantitative experiments on the effectiveness of different kinds of light permit the calculation of the precise extent to which the CO compound of the respiratory ferment absorbs different kinds of light, in other words, the calculation of the ferment's spectrum. The spectrum found is that of a heme compound. No substance, regardless of how it can react with CO or any other inhibitor, can have this spectrum unless it has an iron-porphyrin molecular structure.

2. *The Respiratory Ferment.* a. *Reactions with CO, O_2 and CN.* It has long been known that small concentrations of CO poison vertebrates. Claude Bernard (1883) discovered that CO acts by combining with hemoglobin and thus rendering the red blood corpuscles unfit to

transport their quota of oxygen. Animals which do not depend on hemoglobin for the transportation of oxygen are not influenced by *low* concentrations of CO. Claude Bernard (1883) himself, however, stated that one-sixth of an atmosphere of CO prevents the germination of seeds. This experiment has not been confirmed but it has been shown (Linossier, 1889; Wehmer, 1925) that concentrations of CO as high as four-fifths of an atmosphere do have a poisonous effect on seeds and seedlings. This general poisonous effect of CO was independently rediscovered by Warburg (1926; see also Haldane, 1927) who showed further that the inhibitory effect is on respiration, not on anaerobic fermentation (Warburg, 1927), that it is reversible, and that it may be abolished by light.

Of the CO heavy metal compounds only the CO iron compounds are sensitive to light (iron pentacarbonyl, Mond and Langer, 1891; CO hemoglobin, Haldane and Smith, 1896; CO hemochromogen, Anson and Mirsky, 1925a; Krebs, 1928; CO ferrocysteine, Cremer 1928, 1929; CO carbylamine hemoglobin, Warburg, Negelein and Christian, 1929). And of the iron compounds only some iron porphyrins have an affinity for CO comparable with that of the respiratory ferment and the CO compounds of the iron porphyrins alone have a sensitivity to light comparable with that of the CO compound of the respiratory ferment. Furthermore, it has not yet been demonstrated that any iron compound other than an iron porphyrin can combine reversibly with CO in the dark, as the respiratory ferment does. So from the reactions of the respiratory ferment with CO it is probable that the respiration ferment is a heme derivative—a conclusion which in any case is proven by the fact that the spectrum of the CO compound of the respiratory ferment is a heme spectrum.

Since the known heme compounds have a great affinity for CO it seems surprising that so high a concentration of CO is needed to inhibit respiration or the heme catalysis of the oxidation of cysteine. The reason for this is that these inhibitions depend not only on the CO pressure but, unlike normal uninhibited respiration, on the O_2 pressure also (Warburg, 1926, 1927; Warburg and Negelein, 1928a).

The ferment presumably acts as a catalyst by being oxidized by oxygen and then reduced by substances in the cell the oxidation of which in the procedure constitutes respiration. On this basis the rate of respiration depends on the concentration of the oxidized ferment. In the absence of CO the ferment is practically completely oxidized even when the oxygen pressure is low, so that increasing the O_2 pressure cannot increase the concentration of the oxidized ferment and hence the rate of respiration. In the presence of CO, CO and O_2 compete for the reduced ferment and so the concentration of the oxidized ferment, and hence the rate of respiration, depends on the CO/O_2 ratio according to the equation

$$\frac{[\text{Oxidized ferment}]}{[\text{CO ferment}]} \times \frac{[\text{CO}]}{[O_2]} = K = 10$$

Since the ferment has such a great tendency to become oxidized, the fact that CO can inhibit respiration at all indicates that the respiratory ferment like other heme compounds has a high affinity for CO.

It is a familiar fact (Douglas, Haldane, and Haldane, 1912) that hemoglobin when exposed to a mixture of CO and O_2 reacts reversibly with both these gases according to the mass law equation

$$\frac{[\text{HbO}_2]\ [\text{CO}]}{[\text{HbCO}]\ [O_2]} = K = \frac{1}{100}$$

K here is a true equilibrium constant. Its value means that the affinity of hemoglobin for CO is 100 times as great as the affinity for O_2. It might be supposed from the similarity of the two equations that the respiratory ferment reacts with O_2 and CO exactly as does hemoglobin, the only difference being that the ferment has a relatively greater affinity for O_2 than for CO. This is definitely not the case. For while oxyhemoglobin dissociates spontaneously, this being thermodynamically the reverse of the oxygenation of hemoglobin, the oxidized respiratory ferment is constantly being directly reduced by the oxidizable substances of the cell just as in the heme catalysis of the oxidation of cysteine, the oxidized heme is constantly being reduced by the cysteine which is thus oxidized. The oxidation and reduction of the respiratory ferment are then two different reactions, not simply the same reaction in two opposite directions as in the case of hemoglobin. In addition to direct reduction of the respiratory ferment there may also be spontaneous dissociation, as Warburg supposes. Such spontaneous dissociation of molecular oxygen would not be of use in catalyzing oxidations.

There is, then, not equilibrium in the cell but a steady state in which the ferment is being oxidized by oxygen as fast as it is being reduced. Other things being equal this steady state depends on the concentration of substances which can be oxidized by the ferment. Experimentally, therefore, the K of the ferment is *not* the ratio of two affinity constants but a distribution constant which states what fraction of the ferment in a given steady state is in the oxidized, catalytically active form and what fraction in the carbon monoxide, catalytically inactive form.

It cannot be determined from the mere existence of a distribution constant how the ferment is oxidized and reduced, what the nature and composition of the oxide is, or whether it is analogous to oxyhemoglobin or methemoglobin. The symbol FeO_2 used by Warburg to denote the oxidized ferment is purely arbitrary and should not be taken to mean that the ferment has been proven to have the composition FeO_2.

The inhibition of respiration by CN is not influenced by the O_2 pressure; which indicates that CN combines with the already oxidized ferment and so does not compete with O_2 for the reduced ferment (Warburg, 1927). In harmony with this is the fact that in *neutral* solution CN has a great affinity for oxidized but not for reduced heme compounds (Anson and Mirsky, unpublished experiments). It is not yet proven beyond doubt that CN combines with the same substance in the cell as does CO.

Heme can catalyze the oxidation of many substances (Robinson, 1924; Harrison, 1924; Krebs, 1928, 1929; Kuhn and Meyer, 1928, 1929). Most of these catalyses are inhibited by CN but not the oxidation of olive oil (Kuhn and Meyer, 1929). Similarly the catalysis of the oxidation of linseed oil by hemoglobin is not sensitive to CN (Robinson, 1924). It is also known that the respiration of the plant Chlorella is not inhibited by CN unless glucose is added (Warburg, 1919; Emerson, 1927; Genevois, 1927a, 1927b). And it has been claimed that although the respiration of yeast and other cells is completely inhibited by CN that of some tissues is inhibited by CN to only sixty per cent. The remaining respiration has a respiratory quotient of 0.6 instead of the normal 0.85 (Dixon and Elliott, 1929). It has been suggested (Kuhn and Meyer, 1929) that all these facts fit in with the hypothesis that the oxidation of carbohydrate but not of fat is inhibited by CN and that the uninhibited metabolism of Chlorella and some animal tissues is a fat metabolism. Perhaps the oxidized heme ferment even when combined with CN can still oxidize fat. It is clearly desirable that the supposed cases of the incomplete inhibition of respiration by CN be confirmed and that the effect of CO on the respiration of Chlorella be determined.

Although the oxidation of substances like linseed oil is not inhibited by CN when the catalyst is heme or methemoglobin, when the catalyst is inorganic iron, CN produces a complete inhibition of the oxidation. This is a striking example of the differences in the properties of iron which result when the iron becomes a part of an organic complex. On the basis of the old iron theory, the fact that a catalysis of oxidation is not inhibited by CN would incorrectly be taken as proof that the catalysis was not an iron catalysis.

It has long been known that many tissues give a blue color with the Nadi reagent, which is the so-called indophenol oxidase reaction. This oxidase reaction is inhibited by CO and CN exactly as is normal respiration, so that the heme ferment of normal respiration is probably also involved in the artificial oxidase system (Keilin, 1929). According to Harrison (1929) the ferment produces hydrogen peroxide and this peroxide plus peroxidase are what give the indophenol reaction.

b. *The significance of spectra.* The spectrum of a substance is an expression of the degree of absorption of light of every wave length. This information is best given in the form of a curve in which absorption is plotted against wave length (fig. 1). Usually only the *shape* of the curve is measured, that is, the *relative* extents to which different kinds of light are absorbed. A complete description of the spectrum, however, demands also a statement of the *absolute* amount of light absorbed per molecule of pigment. Pigments of similar chemical structure yield spectrum curves of the same general shape and of similar absolute dimensions. If two pigments have identically the same spectrum then they are identical substances. Substances may thus be characterized or identified by their spectra just as accurately and certainly as by their chemical compositions or melting points. This optical method has the great advantage that it does not necessarily involve the isolation and purification of the substance.

c. *Theory of the new spectrum analysis.* (Warburg, 1927; Warburg and Negelein, 1928d; Warburg, Negelein and Christian, 1929.) In general the amount of light absorbed by a solution of a pigment may be estimated in two different ways. The intensity of the light before and after it has passed through the solution may be measured directly with suitable physical instruments. Or the extent of some specific chemical change in the pigment resulting from the absorption of light may be used as a measure of the amount of light which has been absorbed. The direct physical method requires a solution of pigment free of other pigments and concentrated enough to have a measurable absorption. Clearly the physical method is out of the question for a ferment still present in the cell in minute concentration together with many other substances which absorb light. If the spectrum of the respiratory ferment is to be found it must be by the indirect chemical method, by the

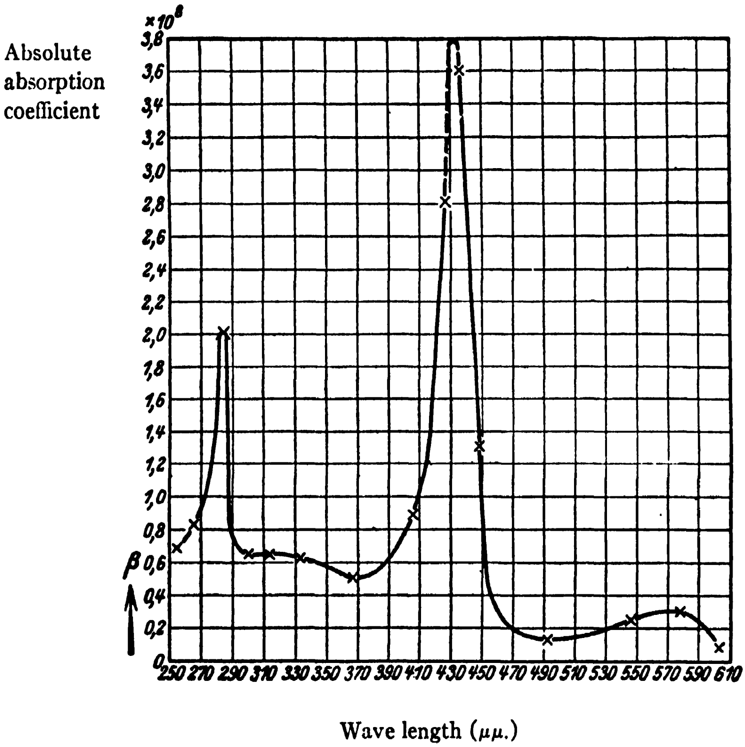

FIG. 1. Absorption spectrum of the respiration ferment

use of the only known specific chemical effect of light on the respiratory ferment, namely, its effect in dissociating the compound of the ferment with CO. The amount of CO which reacts with the respiratory ferment is too small even to be detected. What can, however, be determined experimentally is the rate of respiration. If one assumes that the rate of respiration is directly proportional to the concentration of ferment which is not combined with CO, then the effect of light in increasing the rate of the inhibited respiration is a direct and magnified measure of the CO split off by the light. Light absorbed by other cellular constituents does not influence respiration by splitting off CO from the respiratory ferment and so is not measured. In the absence of CO light has no effect on respiration.

What must be known now is the relation between the rate at which CO groups are split off by a given kind of light, determined by measurements of respiration, and the extent to which the pigment absorbs light; in other words, its spectrum. It may be seen at once that the more strongly the CO pigment absorbs light, the more rapidly will CO groups be split off as a result of the absorption of light. More precisely, the rate at which CO groups are split off is equal to the rate at which light is absorbed times the number of CO groups split off by a unit amount of light. The desired absolute absorption coefficient, β, defines the rate of light absorption by each molecule of the pigment, FeCO, when the light intensity, i, is unity. The rate of absorption of light per unit volume is therefore $i\ \beta\ [\mathrm{FeCO}]$. To know how much light it takes to split off one CO group and how this value varies with the frequency of the light, one must make the simplest assumption, that the reaction obeys Einstein's law of photochemical equivalence. This law states that regardless of the frequency of the light, for every *quantum* of light absorbed a definite constant number, E, of CO groups is split off. The rate at which CO groups are split off, $\frac{d[\mathrm{FeCO}]}{dt}$, is therefore equal to the number of groups split off per quantum of light absorbed, E, times the rate at which quanta are absorbed. This rate is equal to the rate at which light energy is absorbed, $i\ \beta[\mathrm{FeCO}]$, divided by the amount of energy in one light quantum ($h\nu$, h a constant, ν the frequency of the light). So

$$d\,[\mathrm{FeCO}]/dt = E\,\frac{i}{h\nu}\,\beta\,[\mathrm{FeCO}]$$

If i is kept sensibly constant, that is to say, if only a small part of the light is absorbed, then $E \frac{i}{h\nu} \beta$ is a constant, V, and

$$\frac{d\,[\mathrm{FeCO}]}{dt} = V\,[\mathrm{FeCO}] \qquad V = E\,\frac{i}{h\nu}\,\beta$$

V is simply the velocity constant of the reaction. For a different light intensity and frequency, we have

$$V' = E\,\frac{i'}{h\nu'}\,\beta'$$

and

$$\frac{V}{V'} = \frac{i}{i'}\,\frac{\nu'}{\nu}\,\frac{\beta}{\beta'}$$

In order, therefore, to learn the absolute absorption coefficient, β, it is necessary to know the absolute value of E and the V for a given intensity and frequency of light. The measurement of both these values is difficult experimentally. But in order to obtain simply the shape of the absorption curve, that is to say, the relative extents to which different kinds of light are absorbed $\frac{\beta}{\beta'}$, it is necessary to measure only the ratio of two velocity constants $\frac{V}{V'}$, which is relatively easy, and to know that E is some constant, that Einstein's law is obeyed.

d. *The relative spectrum* (Warburg, 1927; Warburg and Negelein, 1928b, 1929b). $\frac{V}{V'}$, and hence $\frac{\beta}{\beta'}$ may be calculated from the effect of light in increasing the constant respiration in the presence of CO, that is, from the effect of light on the distribution constant K:

$$K = \frac{[\text{Oxidized Ferment}]}{[\text{CO Ferment}]}\,\frac{[\mathrm{CO}]}{[\mathrm{O_2}]}$$

$$= \frac{[\text{Oxidized Ferment}]}{[\text{Total Ferment}] - [\text{Oxidized Ferment}]}\,\frac{[\mathrm{CO}]}{[\mathrm{O_2}]}$$

The concentration of the oxidized ferment under any conditions is proportional to the rate of respiration. Since in the absence of CO all the ferment is oxidized, respiration in the absence of CO is a measure of the total concentration of ferment. So K is obtained by measuring the respiration both in the presence and the absence of CO. From the K's for two different kinds of light it is possible to calculate $\frac{V}{V'}$, for the rate of respiration and hence K depends on how much of the ferment is combined with CO. Other things being equal this depends on the ratio of the velocity constant of dissociation of the CO ferment compound to the velocity constant of combination of ferment with CO. In the dark there is only spontaneous dissociation so that the ratio is $\frac{V_{\text{spontaneous dissociation}}}{V_{\text{combination}}}$. In the light there is both spontaneous dissociation and dissociation due to the absorption of light so that the ratio becomes $\frac{V_{\text{spontaneous dissociation}} + V_{\text{light dissociation}}}{V_{\text{combination}}}$. From these two ratios one can calculate $\frac{V_{\text{light dissociation}}}{V_{\text{combination}}}$. If light of another wave length is used one obtains $\frac{V'_{\text{light dissociation}}}{V_{\text{combination}}}$. The velocity constant of combination remains the same since it is not affected by light. By elimination of the combination constant one finally obtains $\frac{V}{V'}$ and hence the desired $\frac{\beta}{\beta'}$.

It is impossible to calculate an individual absolute velocity V, and hence the absolute absorption coefficient, β, from K because K represents a steady state. A steady state is the result of a balance of reactions. An infinite number of combinations of individual velocities could give the same balance and hence the same K.

The relative spectrum of the respiratory ferment obtained in the way just described has the characteristic bands of CO heme compounds. The same spectrum is obtained for different tissues, for different substances being burned, and for different extents of inhibition by CO and about the same degree of inhibition is obtained in a given presssure of carbon monoxide under these different conditions (Warburg, 1929a; Warburg and Negelein, 1929c). Such reasonable and consistent results could hardly be obtained were the method and the assumptions underlying it seriously incorrect.

It is remarkable that when the ferment system of respiration is extracted from the cell or at least when the attempt is made to extract it, the catalytic system in the extract seems more complicated rather than more simple than it is in the cell. The rate of oxidation in the extract and the sensitivity of the catalysis to inhibition are variable; they change with time and with the nature of the substance being burned. From the fact, therefore, that many so-called oxidases may be obtained from cells by different procedures, one cannot conclude that this great variety of oxidases actually exists in the normal cell. It is indeed of great advantage to the cell to have the oxidation of many metabolites catalyzed by the same ferment (Warburg, 1929a).

Further evidence in favor of the soundness of the procedure for obtaining the relative spectrum is given by *in vitro* experiments with known substances. The oxidation of cysteine to cystine is catalyzed by nicotine hemochromogen and this catalysis is inhibited by carbon monoxide (Krebs, 1928). The spectrum of nicotine hemochromogen may therefore be learned in precisely the same manner as was the spectrum of the respiratory ferment by studying the effect of light on the CO inhibition of the hemochromogen catalysis of the oxidation of cysteine. In this case, however, the spectrum may also be ascertained by direct physical measurements of the absorption of light by solutions of nicotine hemochromogen. There is complete agreement in the spectra obtained by the two independent methods (Warburg and Negelein, 1928c). This agreement justifies the two assumptions underlying the method, that the catalysis is proportional to the concentration of oxidized heme and that Einstein's law is obeyed.

e. *The absolute spectrum* (Warburg, 1928d, 1929a; Warburg and Negelein, 1929b). It has been shown that β, the absolute absorption coefficient, is related to the velocity with which CO groups are split off from combination with a pigment because the more the pigment absorbs light, the faster will CO groups be split off as a result of this absorption. In the form of equations:

$$\frac{d\,[FeCO]}{dt} = \frac{E\,i\,\beta}{h\nu}\,[FeCO]$$

$$= V\,[FeCO]$$

$$V = E\,\frac{i}{h\nu}\,\beta$$

$\frac{i}{h\nu}$ may always be measured by direct physical methods. In the case of known solutions of FeCO pigments, β may also be measured by direct physical methods. V may be obtained from the rate at which the pressure in a closed system changes as CO molecules are liberated. In this way E, the number of CO groups split off when one quantum of light is absorbed may be calculated. For a variety of FeCO compounds, iron pentacarbonyl (see also Eyber, 1929) CO ferrocysteine and CO hemochromogen, it is found that a single molecule of CO is liberated for each quantum of light absorbed. It takes the absorption of four quanta, however, to cause the dissociation of one molecule of CO from CO carbylamine hemoglobin. (Warburg, Negelein and Christian, 1929.) Once E is known the β of the respiratory ferment may be obtained from V. In other words, V and $\frac{i}{h\nu}$ are measured and they give the product E β. In the case of known pigments E is obtained from β. In the case of the respiratory ferment β is then obtained from E.

Perhaps the most brilliant part of Warburg's investigations consists in the overcoming of the serious difficulties involved in the measurement of V for the respiratory ferment. It is not feasible to measure accurately the rate at which respiration changes as CO groups are split off when the carbon monoxide derivative of the ferment is exposed to a definite intensity of light. But the difficult problem of measuring the changing respiration of yeast in a constant light has been reduced to the simpler problem of measuring the constant respiration of yeast in a changing light. When yeast whose respiration is inhibited by CO is exposed to light the rate of respiration rises until it reaches the value of the constant respiration in light. When the light is turned off the rate of respiration drops until it reaches the value of the constant respiration in the dark. The total respiration of the yeast in the whole period of oscillation of the light clearly depends on the values of the constant respiration in the dark and in the light (both of which can be measured separately) and on the absolute rates at which the CO groups combine with and become detached from the ferment molecule. Other things being equal the total respiration in a period of oscillation depends on V. The remarkable fact which has been demonstrated theoretically and experimentally is that when the light is made to oscillate faster and faster, a point is reached where the respiration is sensibly constant and not influenced by further increases in the rate of oscillation, and yet is still a function of V. It is from the value of this constant respiration in rapidly oscillating light that V and hence β are obtained.

f. *Peculiarities of the spectrum.* Recently the ultra-violet spectrum of the respiratory ferment has been measured (Warburg and Negelein, 1929b). The striking fact appeared that the respiratory ferment has a marked band in the ultra-violet at about 2700 Å which is lacking in all the pure heme compounds. Proteins, however, due to their tryptophane groups have a characteristic band in the ultra-violet with a wave length of maximum absorption corresponding to that of the respiratory ferment. Although the absolute absorption coefficients of proteins at 2700 Å vary with their tryptophane contents their order of magnitude is the same as that of the respiratory ferment. The simplest conclusion is that the heme of the respiratory ferment is joined to some nitrogenous group probably to a protein, that the respiratory ferment is a hemochromogen. Unfortunately many substances have bands in the ultra-violet, so that the hemochromogen nature of the respiratory ferment may not yet be taken as proven by its spectrum.

It follows from the fact that light absorbed by the non-heme part of the ferment influences the reaction with CO by which the spectrum is measured, that this non-heme part influences the reactivity of the ferment iron, and probably its catalytic reactivity, just as globin influences the properties of hemoglobin's iron (Anson and Mirsky, 1925a). In harmony with this view are the experiments which show that heme only when joined to a hemochromogen-forming nitrogen group is a very effective catalyst of the oxidation of cysteine and that the CO compound only of the hemochromogen is very sensitive to light. Heme itself not combined with a nitrogen group is only a feeble catalyst and the CO compound of free heme has only a small sensitivity to light (Krebs, 1928).

Although the visible, unlike the ultra-violet spectrum of the respiratory ferment, does not show any striking difference from the spectrum of ordinary CO reduced heme prepared from hemoglobin, still the bands are definitely more towards the red end of the spectrum than those of ordinary CO reduced heme (Warburg and Negelein, 1928d). They are much closer to the bands of CO chlorocruorin (Warburg, 1929c). The significance of this shift to the red is not known. It may be due to a partial precipitation of the pigment, to a difference in the heme similar to that of chlorocruorin, to the influence of a peculiar nitrogenous substance, or perhaps to a yet undiscovered inaccuracy of the technique.

g. *The concentration and effectiveness of the respiratory ferment.* The reactions of the respiratory ferment with CO and O_2 permit not only an exact knowledge of the ferment's spectrum but also a rough estimate of the ferment's concentration. A maximum value to the amount of ferment iron is fixed by the amount of CO absorbed in the complete inhibition of respiration. This is so small that it cannot be measured at present. The sensitivity of the procedure is such that this means that in the case of baker's yeast the ferment iron cannot be more than 4×10^{-7} grams per gram of yeast, which is about one two hundred fiftieth the total iron (Warburg and Kubowitz, 1928) and a small part even of the total heme iron. A minimum value of roughly 3×10^{-8} grams iron per gram of yeast can be calculated if one assumes that every collision between a ferment iron atom and an oxygen molecule results in a combination and in the consumption of one molecule of oxygen in respiration (Warburg, 1929b). The minimum and maximum values are both of the same extremely low order of magnitude.

From the approximate value of the concentration of the ferment iron it follows that one atom of ferment iron "activates" very roughly ten thousand molecules of oxygen per minute. To do this the ferment iron must react with oxygen very rapidly. Independent evidence of such a rapid reaction is provided by the fact that the rate of normal respiration is the same in pure oxygen as in pressures of oxygen as low as 10^{-5} atmospheres (Warburg, 1929b). The ferment iron reacts so rapidly with oxygen that it is kept oxidized even when the pressure of oxygen is low so that raising the pressure can have no further effect. The only reason the O_2 pressure is important in CO inhibition is, as has already been stated, because the CO competes with the O_2 for the reduced iron.

h. *Relation to cytochrome.* The respiratory heme is present in too small a concentration to permit of direct spectroscopic detection. This means that the ferment is not identical with the heme pigments which have actually been observed. In any case the components of cytochrome are excluded for under normal conditions they do not combine with CO. It is a curious fact that the iron which has finally been proven to be concerned in biological respiration is not the iron which has always been estimated or the iron which has always been seen.

3. *The Function of Cytochrome.* The question naturally arises what the function of cytochrome is. Experimentally nothing is known. Some properties of cytochrome are known qualitatively; it is known for instance that it can be oxidized and reduced. But what cytochrome actually does accomplish in normal respiration still remains to be demonstrated. Several theoretical possibilities have been suggested (Keilin, 1929; Dixon and Elliott, 1929). It is of course conceivable that the ferment which combines with CO is only one catalyst in a complicated catalytic system of which cytochrome and glutathione are essential parts. There is, however, as yet no adequate experimental basis for a discussion of the question.

The heme pigments have been given credit for the peroxidase action of cells (Keilin, 1929) on the grounds that all hemes are to some extent peroxidases and that the peroxidase action of tissues is in a general way parallel to their content of visible heme pigments. There are, however, so many substances whose concentrations go with the intensity of tissue metabolism that parallelism of concentration is hardly sufficient evidence of identity of structure.

It has also been claimed (Keilin, 1925, 1929) that cytochrome is a respiratory pigment because it can be oxidized and reduced. The chromotologists have always called every pigment which can be oxidized or reduced a respiratory pigment—why, it is not clear. There are many colorless substances in the cell which can be and probably are oxidized and reduced. This property is not taken as proof that they are respiratory substances.

CONCLUSION

It is clear from the investigations which have been discussed in this review that apart from the familiar hemoglobin and chlorophyll, there are many other porphyrin compounds in nature, in all plants and animals. What the functions of these pigments are, is with one exception, unknown, despite the fact that some of them are present in such concentration that their spectra may be observed directly. The one pigment whose function is known is an iron porphyrin, a heme compound which is an essential catalyst of respiration. It is remarkable that even the existence of this heme has been discovered, no less its function. For the heme ferment of respiration is present in only one part in a hundred million. Isolation of such a minute amount of ferment seems out of the question. And the ordinary methods would not permit of the identification of the nature of such a small amount of substance even if it could be isolated. Fortunately the actual measurement of the ferment's spectrum by the carbon monoxide inhibitor technique depends on the results of the presence of the ferment which are great despite the ferment's small concentration. The demonstration of the heme nature of the respiratory ferment is all the more striking when one considers that on the one hand, the chemical nature of no other intracellular enzyme present in minute amount is known, and that, on the other hand, extremely little is known about the normal function of even the ordinary, well studied constituents of the cell which are present in large amounts.

The universal distribution of the porphyrin pigments and the heme nature of the respiratory ferment have been discovered primarily because hemoglobin and its derivatives have been so readily available for study and because these substances have such well defined spectroscopic properties. Once, for example, the reactions and spectra of the hemoglobin derivatives were known, there was no difficulty in identifying cytochrome as a heme compound. More particularly, once the characteristics of the hemochromogens were understood, it became possible to show that the cytochrome complex is a mixture of hemochromogens. Similarly, Warburg's beautiful method for obtaining the spectrum of the respiratory ferment depends on a knowledge of the reactions of the heme pigments with carbon monoxide and of the spectra of these carbon monoxide compounds.

A great deal remains to be found out about the heme pigments. One wonders what the physiological significance is of the close chemical relationship between chlorophyll and heme. Nothing, moreover, is known about the function of cytochrome. And relatively little is known about the effect on the catalytic activity of heme of modification of the porphyrin nucleus and of combination of heme with various nitrogenous substances. It is to be hoped, finally, that the inhibitor technique in the new photochemical form which was devised as a result of the peculiar properties of the heme ferment of respiration will now make possible the discovery of the spectra of other enzymes.

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CULTIVATION AND CLASSIFICATION OF "BACTEROIDES," "SYMBIONTS," OR "RICKETTSIAE" OF BLATTELLA GERMANICA

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The writer[1] recently reported the isolation and cultivation of the intracellular microorganism within the large American roach, *Periplaneta americana*, and found that it was a bacterium belonging to the genus *Corynebacterium* (the diphtheroids). Three types could be distinguished on the basis of certain morphological and serological characters. The species was named *Corynebacterium periplanetae* nov. sp. variety *americana*.

It seemed important to determine whether other closely affiliated insects harbor similar intracellular parasites, and whether the parasite can be adapted to artificial media by using the technical procedures already described. The German roach or "croton bug" (*Blattella germanica*) was selected for investigation.

Sections through the eggs, embryos, and body of *Blattella* show the existence of bacteriocytes with an arrangement and morphology similar to those of *Periplaneta*. The cytoplasm of these cells is packed with bacteria-like microorganisms which may be liberated by triturating fresh tissue in physiological salt solution. As in the case of *Periplaneta*, they are non-motile, non-encapsulated bacilli which are not acid-fast but Gram-positive. The microorganisms stain well by Giemsa's method and are seen to be extremely pleomorphic, with equal and unequal division or budding. The bacilli are straight or crescent-shaped, banded or bipolar, and often terminate in a thickening or club. Short, oval, and coccoidal forms are also common, and appear to represent buds produced from the longer rods. Spores have never been observed. Chains of three units are sometimes seen. The individual units of the bacillary forms have a mean length of approximately 2.5μ with a modal length of 3.2μ. The breadth varies from 0.8 to 1.6μ. The diameter of the coccoidal forms was fairly constant, approximately 0.8μ. The above enumerated facts undoubtedly place the species in the genus *Corynebacterium*.

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[1] Glaser, R. W., *J. Exp. Med.*, 1930, 51, 59.

The procedures for isolating *Corynebacterium periplanetae* free from contaminants and for cultivating the species on dextrose horse blood agar by means of the "spotting technique" have been fully described. The same methods were adopted for the *Blattella* parasite which was isolated and cultivated from the bacteriocytes of six adults and from four embryos removed from egg capsules. Altogether 86 spots were made from these and 10 cultures obtained. Approximately 12 per cent of the attempts succeeded.

The cultivated form corresponds closely to the intracellular parasite. All cultures are non-motile, non-encapsulating, non-sporulating bacilli not acid-fast but Gram-positive. Giemsa's stain shows the same characters previously mentioned, namely, pleomorphism, banding, flexing, with club, oval, and coccoidal forms. In addition cultures on dextrose horse blood agar show many small cocci, diplococci, and *Rickettsia*-like stages. The purity of these cultures can hardly be questioned because they have been repeatedly plated and always yield, in 120 hours; one type of clear, round colony of pinpoint size.

Serological tests with the ten *Blattella* isolations demonstrated the presence of two strains. These strains and their intracellular forms were compared with the *Periplaneta* intracellular parasite and its three cultural types. A morphological differentiation between the intracellular forms was not possible.

Table I represents a series obtained by making 25 measurements for each character. The preparations were fixed in equal parts of absolute alcohol and ether for 15 minutes and stained according to Giemsa's method.

The sizes of the cultural forms reasonably correspond to the sizes of their intracellular counterparts. Slight differences in the mean length of the bacilli occur, but the modal length of the intracellular forms, whether from ova or from mature bacteriocytes, is fairly constant. The cultures show some slight differences from the intracellular forms and from one another. A perusal of both the mean and modal length of all bacillary forms shows that Type II from *Periplaneta* is the longest and Type III from the same host the shortest. Other differences between the three *Periplaneta* types were pointed out in the earlier work.

Strains I and II from *Blattella* are indistinguishable from one another on the basis of size, but may be segregated from the *Periplaneta* cultural types by the large number of minute coccoidal forms which appear in cultures of the former.

TABLE I

Source of parasites	Length range	Mean length	Modal length	Breadth range	Diameter range of coccoidal forms
	μ	μ	μ	μ	μ
Periplaneta bacteriocytes...........	2.0–5.0	3.0	3.2	0.5–1.0	0.5–1.0
Blattella "	1.6–4.0	2.5	3.2	0.8–1.6	0.8
Periplaneta ova..................	0.8–4.8	1.9	3.2	0.5–1.0	0.5–1.0
Blattella "	0.8–4.8	2.4	3.2	0.8–1.2	0.8
Periplaneta cultures:					
Type I....................	0.5–3.2	1.9	3.2	0.4–0.6	0.5–1.0
" II....................	1.6–4.8	3.4	4.0	0.5–1.0	0.5–1.0
" III....................	1.6–3.2	2.1	2.4	0.4–0.6	0.3–0.8
Blattella cultures:					
Strain I....................	0.8–4.8	2.6	3.2	0.4–0.8	minute–0.8
" II....................	1.6–4.0	2.8	3.2	0.8–1.2	minute–1.2

The *Blattella* strains cannot be differentiated from those of *Periplaneta* culturally nor on their fermentation reactions to carbohydrates as Table II shows. None of the forms produce gas. They ferment glucose, sucrose, and maltose, but not lactose nor mannite.

Table III represents some agglutination tests with *Blattella* Strains I and II against normal rabbit serum and against rabbit serum immunized against the three *Periplaneta* types.

These tests were performed at the same time as those described in the previous work, so that a comparison is possible. Such a comparison shows that the *Blattella* strains are distinct from those of *Periplaneta.* Serologically Strain II from *Blattella* resembles Type III from *Peri-*

TABLE II

Carbohydrates	Glucose	Lactose	Sucrose	Maltose	Mannite
pH of medium	7.3	7.3	7.3	7.3	7.3
Periplaneta:					
Type I	5.2	7.3	5.2	5.3	7.4
" II	5.2	7.3	5.0	5.3	7.4
" III	5.4	7.2	5.6	5.3	7.3
Blattella:					
Strain I	5.4	7.3	5.5	5.4	7.3
" II	5.1	7.2	5.5	5.3	7.3

TABLE III

Agglutination Tests

Normal rabbit serum and *Periplaneta*, Types I, II, and III immune rabbit sera against the *Blattella* diphtheroid, Strains I and II. Suspensions of microorganisms in 0.20 per cent NaCl solution. Standardization on Gates turbidity scale to 2.0.

Serum immune to	Titer of immunizing culture	Antigen used	Serum dilutions									NaCl
			1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1,280	1/2,560	
Periplaneta:		*Blattella:*										
Type I...	1/160	Strain I	–	–	–	–	–	–	–	–	–	–
		" II	++	++	+	+	–	–	–	–	–	–
" II...	Clumps spontaneously	Strain I	–	–	–	–	–	–	–	–	–	–
		" II	–	–	–	–	–	–	–	–	–	–
" III...	1/10,240	Strain I	–	–	–	–	–	–	–	–	–	–
		" II	C	C	C	+++	++	++	+	+	±	–
Normal serum		Strain I	–	–	–	–	–	–	–	–	–	–
		" II	–	–	–	–	–	–	–	–	–	–

planeta more closely than the other forms. Distinctions also exist between the two *Blattella* forms.

For the species of intracellular diphtheroid found within *Blattella germanica*, the writer would like to propose the name *Corynebacterium blattellae* nov. sp.

SUMMARY AND CONCLUSIONS

In *Blattella germanica*, the German roach or "croton bug," bacteriocytes are found in all individuals of both sexes. These bacteriocytes are scattered throughout the fat tissue and their cytoplasm is filled with microorganisms. Evidence is presented to show that the intracellular parasites are diphtheroidal bacilli. These diphtheroids are transmitted from one generation to another through the ova.

By using a technic previously described, the intracellular parasites were isolated and cultivated from the adult bacteriocytes and from embryos. Two diphtheroidal strains were cultivated with approximately equal frequency. These two strains resemble one another closely enough to be considered a single species but show certain minor differences. The sizes, general morphology, and tinctorial reactions of the two cultures correspond to the intracellular parasites of *Blattella germanica*. They may be distinguished from the three types of *Corynebacterium periplanetae* variety *americana*, previously described. For the species here discussed the name *Corynebacterium blattellae* nov. sp. is proposed.

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A CONTRIBUTION TO THE EPIDEMIOLOGY OF SPECIFIC INFECTIOUS CYSTITIS AND PYELONEPHRITIS OF COWS

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Plates 28 and 29

(Received for publication, March 7, 1930)

In previous papers (1,2) we called attention to the presence of a specific disease of the urinary tract of cows which had been known in Europe for many years but had not heretofore been recognized in this country. From our material we concluded that the bladder is first involved with secondary involvement of the ureters and kidneys. A bacterium of the diphtheroid type similar to that obtained by several workers in Europe was readily cultivated from the inflamed portions of the urinary tract. In our experiments when small quantities of pure cultures were introduced into the bladder by way of the urethra, a slowly spreading cystitis resulted but the inflammatory process failed to ascend the ureters. Certain bacteriological and other clinical studies failed to confirm the generally accepted view that the urinary infection was associated with genital disease and we were unable to obtain the specific organism from sources other than the urinary tract. Bacteriological examination of the urine of the bulls of two herds in which the disease was prevalent failed to reveal the specific diphtheroid.

It has been difficult to explain the mode of spread within the herd, although certain experiments in which cows were stabled in close contact with others known to be infected resulted in transmission. It was the practice to curry and brush the cows twice a day and probably the infectious material was repeatedly introduced into the vagina by means of brushes. In some other instances the source of infection was not evident. The propagation of the organism seems to lie within the urinary tract of infected cows. Under usual herd conditions transmission thus becomes a difficult matter, since septic material is not usually brushed into the vagina. Some natural means of propagation must necessarily exist and our problem concerned itself with this question.

Dr. Theobald Smith (3), in his examination of the urine of calves, frequently encountered diphtheroids in large numbers, and he has suggested that they might be of significance. It was with the idea of correlating these organisms with those from bladder infections of adult cows that the following studies were made.

Method

In the main the calves were drawn from one herd from which the bulk of our original material had been obtained. A group of the calves were brought to the Institute and used for other experiments, and at autopsy the genito-urinary tract was turned over to us for examination. These were considerably older than the calves from which we obtained material at the abattoir. The calves slaughtered at the Institute varied from 3 weeks to 2 months of age, while those killed at the abattoir were from a few days to 2 weeks old. In a few instances we obtained material from heifers 3 to 4 months old, but most of the material came from males.

The genito-urinary tract was removed in one piece and the whole tract seared. The urine was withdrawn from the bladder and the tract incised from the bladder to the sheath. Cultures were prepared from the bladder mucosa, the upper, middle and lower portions of the urethra, and the sheath, by scraping the mucosa vigorously with a curette and streaking the surface of blood agar plates with the scrapings. The plate cultures were incubated for 48 hours and suggestive colonies picked for inoculation into other media. When material from female calves was examined, scrapings from the bladder, urethra and vagina were made. The urine obtained from the bladder was centrifuged and plate cultures prepared from the sediment. The urinary tracts of 34 calves were examined.

The cultures which conformed in general to the diphtheroidal type were propagated, and, if regarded of sufficient interest, studied on differential media. In this way it was found that several distinct cultural groups existed among the strains. This has been brought out in Table I.

Table I indicates that the diphtheroids fall into 5 cultural groups. It has been shown that the bacterium associated with specific cystitis and pyelonephritis ferments dextrose and fails to ferment the other substances. It produces enough alkali in litmus milk to turn the litmus a deep purple and finally the medium becomes sufficiently alka-

TABLE I

The Cultural Characters of Diphtheroids Isolated from the Urinary Tract of Calves

Group	No. of strains	No. of calves from which isolated	Final hydrogen ion concentration in					Effect on milk	Effect on gelatin	Chromogenesis on potato
			Dextrose	Lactose	Saccharose	Maltose	Mannitol			
I	25	12	4.9–5.3	7.4–8.0	7.4–8.0	7.4–8.0	7.4–8.0	Becomes alkaline; casein dissolved	Not liquefied	Varies from white to faint cream to orange
II	44	18	5.4–5.8	7.6–8.0	6.6–5.0	6.6–5.2	7.6–8.0	"	"	Grayish yellow to orange
III	11	8	5.6	8.0	8.0	6.0	8.0	"	"	Orange
IV	8	3	5.2–5.4	8.0	7.6–8.0	6.8	7.6–8.0	"	"	Cream to orange
V	40	16	7.4–8.0	7.4–8.0	7.4–8.0	7.4–8.0	7.4–8.0	Remains unchanged or becomes alkaline; casein dissolved	"	Yellow to orange

line to dissolve the casein. From Table I it is evident that we succeeded in isolating a large number of strains of proper morphology which possessed the biochemic properties of the strains isolated from the disease. The table also indicates that other types of diphtheroids occur frequently in the urinary tract. Some of them when freshly isolated are readily distinguishable morphologically from those of the first group. Their biochemic features lead to a ready differentiation. It should be stated that all types were non-motile and stained intensely by the Gram method.

The distribution of the various cultural types throughout the genito-urinary tract is of interest and is shown in Table II.

TABLE II

The Distribution of the Various Types in the Genito-Urinary Tract

Group	Isolations from				
	Sheath	Urethra	Bladder	Urine	Vagina
I	16	5	0	3	1
II	7	28	8	1	0
III	1	4	5	1	0
IV	3	4	1	0	0
V	10	11	10	6	3

Cultural Type I, which we regarded as of considerable significance, seems to be confined largely to the sheath. Its entire absence from the bladder is noteworthy although certain strains were recovered from the lower portion of the urethra. On the other hand, the second cultural group seems to live largely in the urethra and bladder, since 36 of 44 strains were isolated from these regions. Group V occurs throughout the urinary tract and has been isolated from the vagina. The other two groups contain such small numbers that their distribution seems to have little meaning.

Since the first group possessed all the morphological and cultural characters manifested by the organism associated with the specific infection in the adults, further correlations were manifestly desirable. With this in view a number of strains were chosen for further study. Rabbits were immunized with cultures isolated from the kidneys of

TABLE III

The Agglutination Affinities of the Group I Strains Obtained from the Urinary Tract of Calves

Culture	Dilutions of serum of rabbit immunized with culture from spontaneous case							Dilutions of serum of rabbit immunized with culture from Calf 1474 sheath							Control
	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:5	1:10	1:20	1:40	1:80	1:160	1:320	
Cow 1132 (left kidney)	C*	C	C	++++	+++	++	++	C	C	C	++	++	+	+	—
Calf 1474 (sheath)....	C	C	C	C	++++	+++	++	C	C	C	C	++++	+++	±	—
" 1494 "	C	C	++++	++++	++	+	—	C	C	+++	++	+	—	—	—
" 1539 (urethra)...	C	C	C	B	B	B	B	C	C	C	B	B	B	B	B
Cow R 418 (urine)....	C	C	C	++++	+++	++	++	C	C	++++	+++	++	+	+	—
Calf 1533 (sheath)....	C	C	C	++++	+++	+++	++	C	C	++	++	++	++	++	—
" 305 (urethra)...	C	C	+++	B	B	B	B	C	C	+++	B	B	B	B	B
" 1540 (urine).....	C	C	C	++++	+++	++	+	C	C	++++	++	++	++	++	+
" 306 (urethra)...	C	C	+++	+++	+++	+	+	C	C	C	+++	++	++	++	+
" 1480 (urine).....	C	C	C	+++	+++	++	++	C	C	C	+++	++	+	±	—
" 303 (sheath)....	C	C	+++	++	+	+	+	C	C	+++	++	++	++	+	—
" 307 "	+++	C	C	+++	—	—	—	C	C	+++	+	—	—	—	—
" 1480 "	++++	+++	+	B	B	B	B	C	C	++	+	B	B	B	B
" A "	C	C	B	B	B	B	B	C	C	+++	B	B	B	B	B
Cow D. M. (urine)....	C	C	C	C	C	C	+++	C	C	C	++++	+++	+++	++	—
Calf 1531 (sheath)....	C	C	+++	+	—	—	—	C	C	++	+	±	—	—	—
" 1522 (urine).....	C	C	C	C	+++	++	+	C	C	C	+++	++	+	+	+

* C = complete. The quantity of agglutination has been recorded as +, a definite but slight clumping, to ++++, a heavy clumping without clearing. B = spontaneous precipitation in the form of a button at the bottom of the tube.

spontaneous cases and with certain typical strains from calves. When sera with sufficient agglutinin were obtained all strains were tested. That these possessed agglutination affinities similar to those of the organisms isolated from the kidneys of adults in spontaneous infections is shown in Table III.

On the whole it can be said that the strains resembling the etiological type behave in a manner similar to those isolated from spontaneous cases in cows. The serum from a typical calf strain is capable of agglutinating those from the cows and *vice versa.*

TABLE IV

The Effect of Reciprocal Absorption on Agglutination

Culture	Serum dilutions of culture Cow 1132 serum absorbed with culture Calf 1474						Serum dilutions of culture Calf 1474 serum absorbed with culture Cow 1132						Control
	1:10	1:20	1:40	1:80	1:160	1:320	1:10	1:20	1:40	1:80	1:160	1:320	
Cow 1132 (kidney)...	±	−	−	−	−	−	+	±	−	−	−	−	−
Calf 1474 (sheath)....	−	−	−	−	−	−	±	−	−	−	−	−	−
" 1494 "	+	+	−	−	−	−	−	−	−	−	−	−	−
" 1539 (urethra)...	+	−	−	−	−	−	−	−	−	−	−	−	−
Cow 418 (urine)......	+	±	−	−	−	−	+	−	−	−	−	−	−
Calf 1533 (sheath)....	+	±	±	±	±	±	+	+	±	±	±	±	±
" 1540 (urine).....	±	±	±	±	±	±	±	±	±	±	±	±	±
" 306 (urethra)...	+	±	±	±	±	±	++	±	±	±	±	±	±
" 1480 (urine).....	+	−	−	−	−	−	±	−	−	−	−	−	−
" 303 (sheath)....	±	±	±	±	±	±	+	±	±	±	±	±	±
" 1480 "	−	−	−	−	−	−	−	−	−	−	−	−	−
" A "	−	−	−	−	−	−	−	−	−	−	−	−	−
Cow D. M. (urine)...	±	−	−	−	−	−	+	+	±	±	±	−	−
Calf 1531 (sheath)....	−	−	−	−	−	−	−	−	−	−	−	−	−
" 1522 (urine).....	±	±	−	−	−	−	±	±	−	−	−	−	−

In the course of the correlation it seemed advisable to test the antigenic specificity further. With this in mind, the serum produced by immunization with the strain obtained from a cow's kidney was absorbed with the culture obtained from the sheath of Calf 1474 and the other serum was absorbed with the culture isolated from the kidney. The absorbed sera were then tested with the various strains as shown in Table IV.

The results of the tests of the sera after absorption indicate that a typical strain obtained from the sheath of a healthy calf is capable of absorbing practically all the agglutinin from the antiserum specific for the etiological strains. The same held true when the serum produced by immunization with one of the calf strains was absorbed with a culture from an adult infection. The cultural and immunological findings support the view that the organisms are similar.

The calves from which cultures were obtained were apparently normal. This brought up the question of pathogenicity. It is known that freshly isolated cultures when injected into rabbits or guinea pigs in considerable quantities fail to produce lesions. Our previous experiments indicated that small quantities of culture from either bladder or kidney infections when introduced into the cow's bladder by means of a catheter gave rise to a slowly progressive persistent cystitis. The most important test of identity seemed to us to be that of the pathogenic properties of the strains. Four cows were available for the tests and these were injected with various cultures. The details are given in the following protocols. The urine from all was examined twice at intervals before the injections were made. Cultures failed to show significant diphtheroids and the urine was free from albumin and other abnormal products.

On April 22, 1929, 4 cows were inoculated intra-urethrally with 3 cc. of 48 hour broth cultures, as follows: Cow 1517, culture from the urine in spontaneous infection of Cow R 418. Cow 1520, culture from the lower urethra of Calf 1539. Cow 1530, culture from the sheath of Calf 1539. Cow 1559, culture from the sheath of Calf 1480.

One week later the urine of all was examined. The urinary sediment of all contained diphtheroids similar in morphology and cultural characters to those inoculated. Six days after the first examination specimens were again examined. The urine from Cows 1517, 1520, and 1530 showed little abnormal; that from Cow 1559 was turbid and gave a strong reaction for albumin. One week later the urine from all 4 cows was reexamined with the same results. The diphtheroids had disappeared from the urinary sediment of 1517, 1520, and 1530. The specimen from 1559 contained blood, gave a strong albumin reaction, and was rich in red cells, clots and diphtheroids. It was at this time that the first urethral hemorrhage was observed. Urination became more frequent and considerable straining following micturation, often accompanied by the passage of bright red blood. A sample of urine obtained 3 months after the inoculation revealed blood, mucus, and large numbers of diphtheroids.

Cow 1559 was slaughtered at an abattoir 6 months and 21 days after inoculation.

The whole genito-urinary tract was brought to the laboratory for examination. The bladder was contracted, its walls thickened, and the vessels of the subserosae dilated. The whole mucosa was congested, swollen and wrinkled, and scattered over its surface there were many irregularly rounded raised hemorrhages. On the floor of the bladder were a number of irregular, slightly raised, flattened, adherent patches which were removed with difficulty, exposing a raw, weeping surface. The mucosa between and about the outlets of the ureters appeared as a wrinkled, greatly swollen, reddened, spongy mass. Both ureters were dilated and the walls markedly thickened. The right was almost half again as large as the left (Fig. 1). The mucosa of both appeared edematous and was streaked here and there with longitudinal red lines. Portions of two lobes of the right kidney contained irregularly rounded, grayish white, homogeneous areas which involved only the cortex. Several irregular, sharply circumscribed accumulations of yellowish green caseous material were present at the tips of the papillae (Fig. 1). The areas varied from 1 to 2 mm. in diameter to nodules as large as 1 cm. (Fig. 2). The caseous material shelled out easily revealing a glistening capsule. Films from the caseous areas revealed enormous numbers of diphtheroids. Portions of the mucosa of the pelvis were swollen and reddened (Fig. 1). The mucosa of the ureter where it left the kidney revealed a similar process (Fig. 1). In addition to these findings a cyst which protruded from the vaginal wall was noted. Diphtheroids were readily obtained from the urine at autopsy. Films from the pelvic abscesses showed diphtheroids in enormous numbers to the exclusion of other forms. Cultures developed only diphtheroids. The left kidney revealed no abnormalities.

Pieces of the bladder, ureter and right kidney fixed in Zenker's fluid revealed well marked changes. Microscopic examination of sections of the bladder showed a variety of lesions. The mucosa in one area was largely intact but the submucosa was greatly thickened, edema was pronounced, accumulations of round cells and hyperplasia of the connective tissue accompanied by capillary proliferation were remarkable. In other portions little of the mucosa remained except for a few streamers of degenerated cells. The exposed surface consisted of a richly cellular submucosa composed of proliferating connective tissue containing many vessels and heavily infiltrated with round cells. The left ureter presented little abnormal except edema of the submucosa and proliferation of the submucous connective tissue. The mucosa of the right ureter revealed degenerative changes and some exfoliation. The deeper portions of the mucosa were invaded by round cells. The submucosa was edematous and rich in capillaries as well as moderately infiltrated with round cells.

The left kidney presented no serious derangement. The section from the right revealed little abnormal in the cortex. The medulla presented no abnormality until close to the papillus, where some of the tubules revealed exfoliation of the mucosa. There was a moderate engorgement of the capillaries. Nearer the pelvis the desquamation was more marked and the vascular engorgement more pronounced. About the tip of the papillus and extending a uniform depth into the kidney a well defined lesion was observed. On the whole the tubular outlines were well preserved but the mucosa had disappeared and the lumina were plugged with homogeneous material. Hemorrhages into the tubules were not infrequent. Under lower magnifications several deep blue staining, clear cut masses lying close to the papilla and having the outlines of large tubules were readily discerned (Fig. 3). These when sufficiently magnified proved to be tubules of which only the basement membrane remained, and the blue staining areas were composed of densely packed masses of diphtheroids (Fig. 4). On the whole the lesion impressed us as being relatively recent and was characterized by its sharp definition and its relative freedom from cellular reaction. The remarkable growth of the causative organism within the tubules with consequent interference with the neighboring circulation and the absorption of bacterial products might well account for its character.

The other 3 cows were killed 24, 39, and 53 days after inoculation. The bladders and kidneys failed to show abnormalities and the organisms could not be recovered from the urine.

It will be seen from the protocols that two of the three cows inoculated with cultures obtained from the lower urinary tract of calves developed only transient infections. The inoculation in the third instance (Cow 1559) produced cystitis, pyelitis and pyelonephritis. The cow injected intra-urethrally with culture from the kidney of a spontaneous infection developed only transient cystitis.

DISCUSSION

The findings indicate that the lower genito-urinary tract of male calves may harbor an organism similar in morphology, cultural characters and certain immunological properties to the organism encountered in this country and Europe in spontaneous infections of the bladder, ureter and pelvic portions of the kidneys of cows. The organism has been found in the sheath, urine, and lower portions of the urethra in 12 of 34 calves examined. By far the greatest number of strains were obtained from the sheath. The mucosa of the sheath and the retained urine habor bacteria in enormous numbers and the diphtheroid seems to make up but a small proportion of the organisms present.

The findings are significant from an epidemiological standpoint. As we have pointed out, the difficulty in tracing infection from animal to animal within the herd has been great. It seems probable that many cases may really originate as infections during early life. The organisms are able to maintain themselves on the mucous membranes of the lower urinary tract where little damage is done, but by a gradual, ascending growth they may finally reach the upper urethra and bladder. Where the tube is short as in the female the supposition is reasonable; and in this sex additional complications, such as pressure on the bladder, albuminuria, etc., may act to quicken the invasive process. Unfortunately our material contained only a few female calves; but from one of these we obtained from the vagina in the region of the urethral orifice a culture indistinguishable culturally and immunologically from the pathogenic type. The source of the fairly frequent sheath infestation is not easily traced. In one instance a calf born of an infected mother was examined and the organism recovered from the sheath. The same organism was readily cultivated from the mother's urine and in all probability the sheath was directly inoculated during parturition. In five other instances the urine of the mothers of calves in which we had cultivated the diphtheroid from the sheath was examined with negative results.

The brief citation of a spontaneous infection in a 12 day old male calf serves as evidence that infection may begin in early life.

The genito-urinary tract was obtained immediately after slaughter. The kidneys presented no gross abnormalities. The bladder walls were greatly thickened and the mucosa swollen and edematous. Hemorrhages varying in size from minute points to considerable extravasations were scattered over the mucosa. The urine within the bladder was turbid and contained blood. The urinary sediment was rich in red cells, round cells, leucocytes, and diphtheroids. Typical diphtheroids were obtained in pure culture. Microscopic examination of fixed and stained material from the bladder revealed the usual type of cystitis encountered in adult infections of this kind.

Certain facts (2) indicate that the sheath of the adult male does not contain many organisms of the etiological type, since we failed to cultivate them from naturally passed urine in 11 instances, although organisms similar to those of Groups II and IV were readily grown.

From the experiment in which 3 cows were artificially infected with strains from calves and only 1 developed the disease, it might be suggested that certain of the calf strains are not pathogenic. It is true that all the cultures employed in the injection had been under artificial cultivation for several months and that that from the spontaneous case was isolated 2 years before the inoculation. The strain which produced the infection in the inoculated cow had been isolated about 1 year before it was used. On the other hand, as a control procedure, a cow was inoculated with a culture obtained from the kidney of a severe case but this gave rise only to transient cystitis. From this it can be argued that the resistance of certain cows might explain our results.

We have produced cystitis in a fair proportion of inoculations, but the disease has thus far been confined to the bladder. In the instance of the cow which we regard as most susceptible (1559) culture introduced into the bladder produced the local process and later reached the pelvis of one kidney by way of the ureter. The experimental evidence indicates that under favorable conditions diphtheroids introduced into the bladder may produce cystitis, pyelitis and pyelonephritis.

SUMMARY

Bacteriological examination of the genito-urinary tract of calves originating in a herd in which infectious cystitis and pyelonephritis exists among the cows, revealed a variety of cultural types of diphtheroids. Of these types, one obtained from a considerable number of the calves resembled in morphology and cultural characters the organism cultivated from the actual cases of the disease. This group had agglutination affinities like those of the organism mentioned and was capable of absorbing agglutinin from antiserum specific for it. When three cows were inoculated intra-urethrally with cultures isolated from the sheaths of calves, two developed transient infections and the other a severe prolonged cystitis and pyelonephritis.

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EXPLANATION OF PLATES

Plate 28

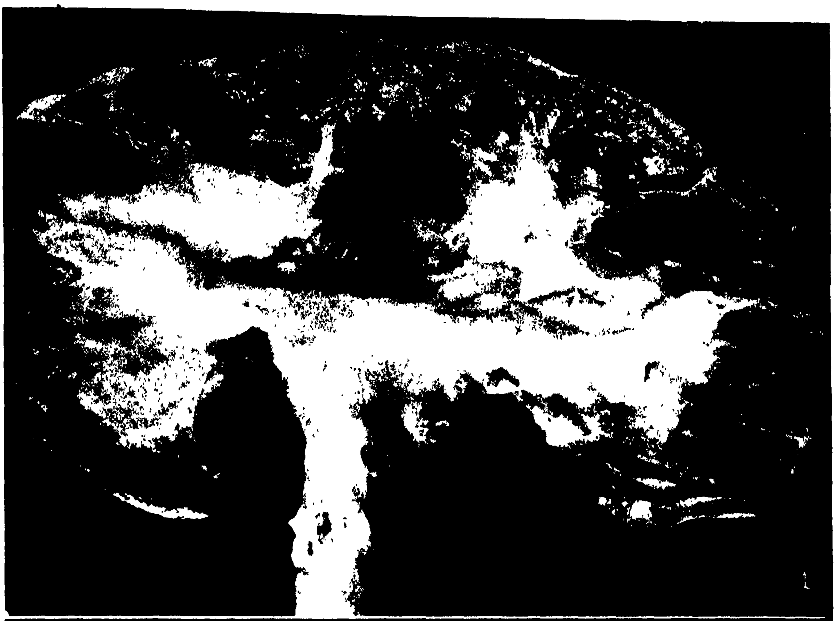
Fig. 1. The right kidney of Cow 1559 which had been inoculated intra-urethrally with culture obtained from the sheath of a normal calf. Note edema and congestion of the pelvic mucosa, the size and appearance of the ureter. The arrows indicate the location of small abscesses. About ⅔ actual size.

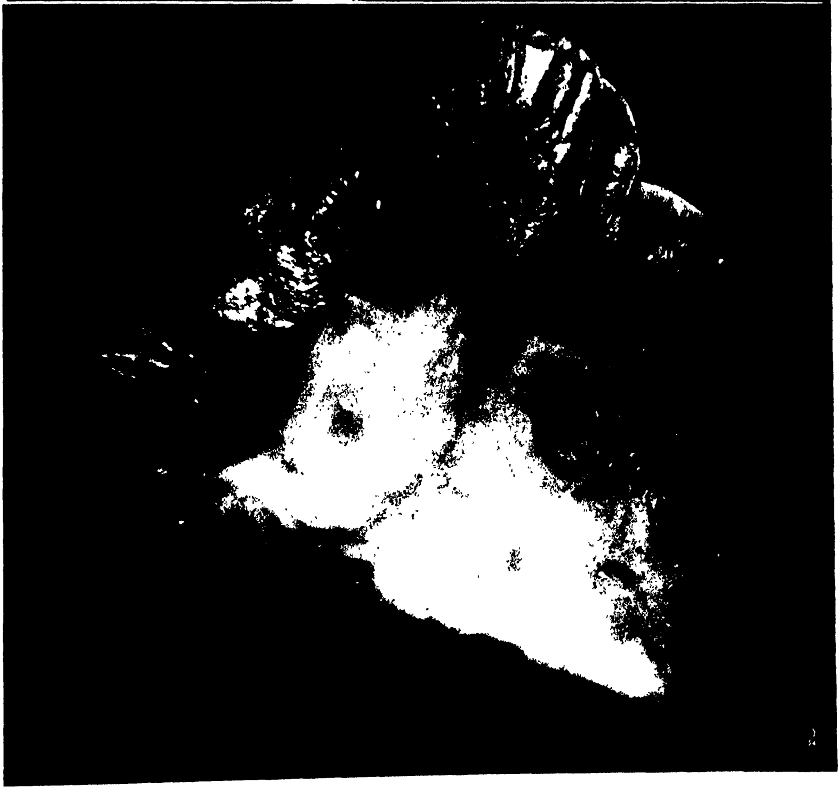
Fig. 2. The largest abscess encountered in another portion of the right kidney. × about 1¼.

Plate 29

Fig. 3. Section of the right kidney of Cow 1559 showing an early lesion deep within the medulla. Note the degenerative changes and the intensely black tubules due to deeply stained bacterial masses within them. Zenker's fixation; eosin and methylene blue stain. ×90.

Fig. 4. A cast composed of tightly packed masses of the diphtheroid from a single tubule from the same preparation. ×660.





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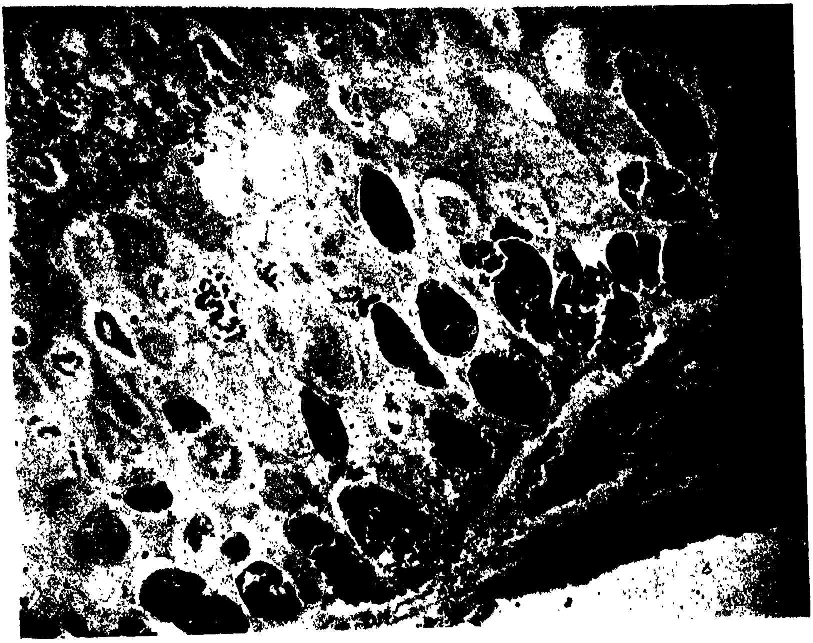

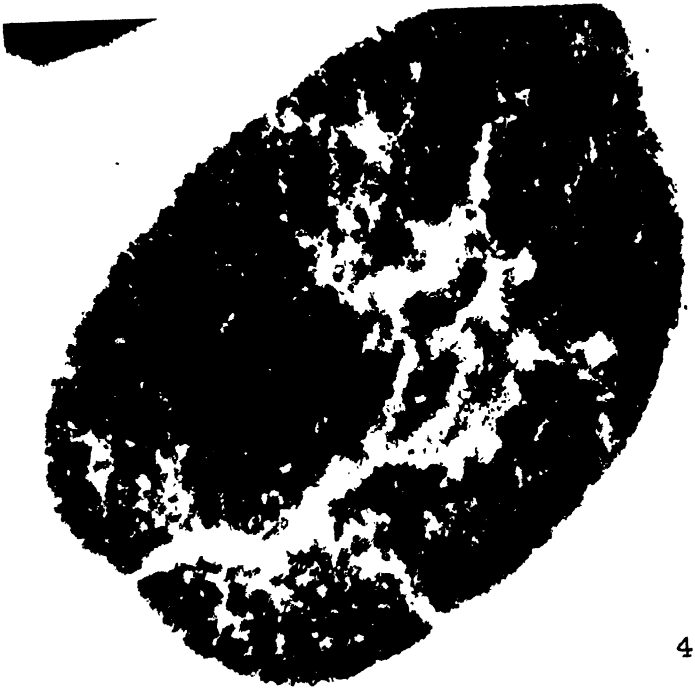

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